PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

IN THE APPLICATION OF:

ANTHONY J. KINNEY ET AL.

CASE NO.: BB1538USNA APPLICATION NO.: 10/776311

CONFIRMATION NO.: 4023 GROUP ART UNIT: 1638

EXAMINER: DAVID T. FOX FILED: FEBRUARY 11, 2004

FOR: PRODUCTION OF VERY LONG CHAIN POLYUNSATURATED FATTY ACIDS IN OIL SEED PLANTS

Via EFS-Web

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

Brief on Appeal

This is an appeal of the Final Rejection, mailed July 16, 2008, of claims 1, 12, 16 and 26 of the above-identified application.

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(I) Real Party in Interest

The real party in interest in this Appeal is E. I. du Pont de Nemours and Company, the assignee of the entire right, title and interest of the above-identified patent application.

(II) Related Appeals and Interferences

There are no related Appeals or Interferences which will directly affect or be directly affected by or have a bearing on the Board's decision in the pending Appeal.

(III) Status of Claims

Claims 1-139 were originally filed.

A Restriction Requirement was issued and the subject matter of Group I, Claims 1, 11, 12, 16-18 and 26-28 were elected for further prosecution. Claims 11, 17, 18, 27 and 28 were cancelled during prosecution. There is one independent claim: 1.

The currently pending and appealed claims are claims 1, 12, 16 and 26 which are set forth in the Claims Appendix attached hereto.

(IV) Status of Amendments Filed Subsequent to Final Rejection

A first Response after Final was filed electronically on March 14, 2008. Finality of the Office Action mailed on February 6, 2008 was withdrawn and prosecution was reopened. A second Response After Final was filed electronically on October 10, 2008. This second Response After Final was not entered as set forth in the Advisory Action re-issued and dated January 21, 2009

(V) Summary of the Invention

The invention on appeal is believed to constitute pioneering work wherein an exogenous DHA and/or EPA biosynthetic pathway was incorporated into an oilseed plant thereby enabling the oilseed plant to produce, for the first time, at

least 1% DHA and/or EPA in the seed oil. This simply was not known prior to Applicants' disclosure.

Claim 1 concerns a transgenic oilseed plant that produces mature seeds in which the total seed fatty acid profile comprises at least 1.0% of at least one omega -3 polyunsaturated fatty acid having at least twenty carbon atoms and five or more carbon-carbon double bonds wherein said transgenic oilseed plant comprises in its genome at least two transgenic nucleic acid sequences encoding at least two different polypeptides and further wherein at least one polypeptide has desaturase activity and at least one polypeptide has elongase activity.

This is discussed in the specification, *inter alia*, on page 17 starting at line 3 through line 2 on page 19, Examples 3-8 and 10-13, and claims 1, 11 and 12 as originally filed.

Claim 12 concerns the oilseed plant of claim 1 wherein the polyunsaturated fatty acid is an omega-3 fatty acid selected from the group consisting of eicosapentaenoic acid (EPA), docosapentaenoic acid (DPA), and docosahexaenoic acid (DHA).

This is discussed in the specification, *inter alia*, on page 4 at lines 17-18, on page 17 starting at line 37 through line 2 on page 18, and Examples11 and 13 and in claim 12 as originally filed.

Claim 16 concerns seeds obtained from the transgenic plant of claim 1 or 12 wherein the seed comprises the transgenes.

This is discussed in the specification, *inter alia*, on page 4 at lines 3-8, pages 24 and 25, and Examples 4 and 5.

Claim 26 relates to the transgenic oilseed plant of claim 1 or 12 being selected from the group consisting of soybean, Brassica species, sunflower, maize, cotton, flax, and safflower.

This is discussed in the specification on page 17 at lines 35-36 and in claim 26 as originally filed.

(VI) Grounds of Rejection To Be Reviewed on Appeal

There are two grounds of rejection presented for review:

a) Whether claims 1, 12, 16 and 26 comply with the enablement requirement of 35 USC §112, first paragraph. Specifically, it is contended that the specification is not enabling for claims drawn to any oilseed plant of any genotype producing the claimed levels of fatty acids.

b) Whether claims 1,12, 16 and 26 are obvious under 35 USC §103(a) over Knutzon et al. (U.S. Patent No. 6, 075, 183 issued June 2000) in view of Abbott Laboratories (WO 02/08401), further in view of each of Mukherji et al. (U.S. 7, 211,656) or Browse et al. (U.S. Patent no. 6,884,921).

(VII) Argument

(a) The rejection of claims 1, 12, 16 and 26 as failing to comply with the enablement requirement of 35 USC §112, first paragraph.

The pioneering work that constitutes the subject matter of the invention on appeal concerns placing an exogenous DHA and/or EPA biosynthetic pathway into an oilseed plant and recovering at least about 1.0:% of DHA and/or EPA in the seed oil. **This simply was not known prior to Applicants' disclosure.**

It is well known that oil seeds are varieties of oil-rich seeds, nuts, fruits, and cereals that are used in vegetable oils and fats for cooking, food manufacture, soap making, specialized lubricating oils, and cosmetics.

Native or wild-type oilseeds do not normally produce mature seeds having the recited oil profile, i.e., at least 1.0% of at least one polyunsaturated fatty acid having at least twenty carbon atoms and five or more carbon-carbon double bonds. Since higher plants, in general, lack the metabolic pathway necessary to synthesize a PUFA having at least twenty carbon atoms from the dietary intake of precursor fatty acids, LA and ALA, it is highly unlikely that this could be achieved via mutagenesis. (Sayanova et al., Phytochemistry 65(2004) 147-158) It appears that the best route to enable higher plants to synthesize a PUFA having at least twenty carbon atoms from the dietary intake of precursor fatty acids, LA and ALA, is to use genetic engineering technology.

A copy of Sayanova et al. was previously submitted and is attached hereto as Evidence Appendix A.

The research which constitutes the subject matter of the instant invention has paved the way for other researchers to use this foundation in engineering the production of omega-3 fatty acids in oilseed crops.

Dr. Kinney's previously submitted declaration dated March 23, 2007 (copy provided as Evidence Appendix B), a copy of an article co-authored by Dr. Kinney and Dr. Howard Damude, the co-inventors of the subject invention (copy provided as Evidence Appendix C), a copy of the Roberts review (copy provided as Evidence Appendix D) were previously submitted to demonstrate that a variety of approaches using different genes and combinations thereof can be used to make transgenic plants producing long chain polyunsaturated fatty acids.

For example, the Kinney Declaration discussed the Robert article specifically, Table 1, on page 105 of the Robert article. This table summarizes genes, host plants and reported LC-PUFA proportions in seeds of transgenic plants:

Column 1 references the work of Abbadi et al. (2004) with respect to flax.

Column 2 references the work of Kinney et al. (2004) (which constitutes the subject matter of the instant application) with respect to soy.

Column 3 references the author's work in 2005 with respect to *Arabidopsis*; and

Column 4 reference the work of Wu et al. (2004 with respect to *Brassica/*rapeseed.

The Robert article then went on to discuss Dr. Kinney's work in column 1 on page 106 (and also mentions the instant patent application and publication). The work of Wu et al. is discussed in column 1, second paragraph. The Robert article states that **Dr. Wu used Dr. Kinney's method to produce high levels of AA and EPA in rapeseed.** Specifically, a delta-17 desaturase was used to shunt a large amount of AA into the n-3 pathway at EPA. **Thus, Dr. Wu's work demonstrates that Dr. Kinney's method worked with respect to *Brassica*.** Irregardless of the LCPUFA oil level, Dr. Wu did exemplify the use of Dr. Kinney's

method to achieve expression of LCPUFAs in *Brassica*, **a different oilseed plant than soy**. LCPUFAs were recovered. This further shows that the specification is indeed enabling.

Furthermore, it is noted that a number of different EPA/DHA genes from different sources are presented in the specification to produce EPA/DHA oils in transgenic oilseeds. It should be clear from the specification that as long as a pathway is capable of producing DHA and/or EPA, then that pathway can be used to produce such fatty acids in seed oils.

Attention is kindly invited to Figure 1 of Damude et al. (Evidence Appendix C). This figure depicts aerobic LCPUFA biosynthetic pathways in marine microbes. In other words, there are a number of pathways available that could lead to the same product. Attention is kindly invited to Damude et al. (2007) second column on the third page through column 2 on the fifth page. This section discusses a variety of fatty acid biosynthetic pathways that can be considered in engineering omega-3 LCPUFAs in plants (also depicted in Figure 1).

Thus, Dr. Kinney's declaration also was intended to demonstrate that a variety of genes (from different sources) and combinations thereof can be used to engineer production of omega-3 fatty acids in oilseed crops. The art cited by Applicant was intended to demonstrate that the instant invention is not limited to any particular combination of enzymes (and genes encoding them). The choice of genes will vary depending on which pathway is chosen, for example, whether a delta-6 or delta-8 pathway is chosen to engineer expression of an LCPUFA in a oilseed plant. The Napier, Wu and Damude articles all support this point. A copy of Napier is provided in Evidence Appendix E and a copy of Wu is provided in Evidence Appendix F.

It was noted in the Response dated April 17, 2008 that certain oilseeds, have various amounts of stearidonic acid (SDA) in their oil. Since SDA is already present in these particular seeds, then a single desaturase along with a single elongase is all that would be needed to convert SDA to EPA using the methods described in the instant application.

Those of ordinary skill in the art know that blackcurrant seed oil is sold over the counter by a variety of companies. It is known to contain about 2-5% of SDA. This information is readily available on a variety of web sites and on packaging/inserts of these products wherever they are marketed. For example, Aromtech markets Ribesin® Blackcurrant seed oil capsules.

With respect to hemp, those of ordinary skill in the art should be aware that there is a www.hempfood.com website that provides nutritional information about hemp seed and hemp seed oil.

There can be no doubt that borage, blackcurrant and hemp are all considered to be oilseeds.

Furthermore, it is noted that a number of different EPA/DHA genes from different sources are presented in the specification to produce EPA/DHA oils in transgenic oilseeds. It should be clear from the specification that as long as a pathway is capable of producing DHA and/or EPA, then that pathway can be used to produce such fatty acids in seed oils.

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To reiterate, Dr. Kinney's declaration also was intended to demonstrate that a variety of genes (from different sources) and combinations thereof can be used to engineer production of omega-3 fatty acids in oilseed crops. The art cited by Appellant demonstrates that the instant invention is not limited to any particular combination of enzymes (and genes encoding them). The choice of genes will vary depending on which pathway is chosen, for example, whether a delta-6 or delta-8 pathway is chosen to engineer expression of an LCPUFA in a oilseed plant. The Napier, Wu and Damude articles all support this point (Evidence Appendices, C, D and E).

b) <u>The rejection of claims 1,12, 16 and 26 are obvious under 35 USC §103(a) over Knutzon et al. (U.S. Patent No. 6, 075, 183 issued June 2000) in view of Abbott Laboratories (WO 02/08401), further in view of each of Mukherji et al. (U.S. 7, 211,656) or Browse et al. (U.S. Patent no. 6,884,921).</u>

It should be noted that the instant application was filed non-provisionally on February 11, 2004 and claims the benefit of priority of a provisional application that was filed on **February 12, 2003**.

The reason that the Qi et al. and Abbadi et al. (both references were cited by Robert, Evidence Appendix D) were cited by Applicants was to demonstrate what has happened in the wake of the pioneering contribution made by Dr. Anthony Kinney and his group in engineering oilseed crops to produce omega-3 fatty acids. **The February 12, 2003 priority date of the instant application demonstrates that Dr. Kinney and his team were at the forefront of engineering oilseed crops to produce omega-3 fatty acids.** (Emphasis added).

For clarification, the following is noted:

a) It should be noted that the Qi et al. reference was cited by Robert on page 104, column 1 of bottom paragraph. A copy of Robert is provided in Evidence Appendix D. Qi et al. was published in June, 2004. It was noted on page 5 of the Office Action mailed on July 16, 2008 that Qi was published prior to publication of Applicant's PCT Application. Applicants kindly noted that the earliest priority date to which the instant application is entitled is February 12, 2003. Thus, the priority date of the instant application predates Qi et al. by well over one year.

b) Abbadi et al. (October, 2004) was published well after the February 12, 2003 priority of the instant application. Abbadi et al. was also referenced by Robert. The same is true of Roberts (2006) and Wu (2005).

The basis of rejection of the claims under 35 USC §103(a) rejection as set forth in the Office Action dated April 1, 2008 was that it "would have been obvious to one of ordinary skill in the art to utilize the method of Brassica transformation with the delta-5 an delta-6 desaturase genes under the control of the seed-specific napin promoter for the production of novel PUFAs in the seed oil of transgenic plants as taught by Knutzon et al, and to modify that method by incorporating the elongase genes taught by ABBOTT LABORATORIES under the control of a seed-specific promoter,and to further modify that method by incorporating the omega-3/delta 17 desaturase genes taught by Mukherji et al or Browse et al under the control of a seed-specific promoter such as the napin promoter for the production of EPA in the oil of the transgenic Brassica seeds, as suggested by each reference."

The cited references do not demonstrate accumulation in seed oil of an oilseed plant of at least 1% DHA or EPA. Further, when taken together the individual enzymatic conversions efficiencies in plants, disclosed in each of the references, are not sufficient to enable an accumulation in seed oil of at least 1% DHA or EPA.

Synthesis of GLA and SDA is a single linear conversion from LA or ALA.

In contrast, EPA and DHA are membrane lipids not normally found in any plant oil. Synthesis of EPA from endogenous plant lipid is not linear. Prior to the instant invention, it was not known if a plant could accumulate these fatty acids in their oil.

Fatty acids are desaturated while part of membrane lipids, they are elongated while attached to acyl-CoA. Thus, fatty acids need to pass in and out of the phospholipids in the plant cell membrane as part of the pathway to synthesizing EPA. This step was thought to be a major block in converting ALA to EPA in plants (as described by Robert in legend to Figure 1 on page 104, second column and page 105, Evidence Appendix D). Consequently, this led researchers to look for acyltransferases involved in the exchange of acyl groups between membrane lipids.

In Wu et al, cited by Robert, the investigators use the same method disclosed in the instant application to make 15% EPA in *Brassica* seeds. When an additional acyltransferase is added to the constructs, there appeared to be no effect on the total EPA content. Robert also describes an attempt by his own group to look for acyl-CoA desaturases from fish and express them in plants in an effort to avoid having fatty acids pass in and out of membrane phospholipids.

It was concluded, however, that this is not necessary and, in the light the information disclosed in the instant application, should essentially use the methods described in the instant application to design LCPUFA pathways.

Likewise, the synthesis of DHA from EPA in higher organisms is a complex series of events involving two acyl-CoA elongation reactions, movement to membrane phospholipids, a membrane desaturation, movement of the resultant fatty acid into the peroxisome and then a beta-oxidation reaction to produce DHA.

Knutson describes the production of GLA and STA. The Abbott, (Biorignal) and Browse patents disclose LCPUFA elongases and desaturases.
GLA and STA are found in the oils of some plants. It is stated in Example 5, column 21 at lines 30-36 that transgenic leaves contained only 0.2 to 0.7 wt % DGLA and noted that DGLA was being converted to ARA in leaves. Expression in seed showed the presence of taxoleic acid and pinolenic acid. These are the expected products of delta-5 desaturation of oleic and linoleic acid.

Knutson states on column 24 at lines 20-26 that three different substrate specificities were expressed in a heterologous system. The polyunsaturated fatty acids produced were the following:

a) ARA (20:4) from the precursor 20:3 (DGLA);
b) production of GLA (18:3) from 18:2 substrate; and
c) the conversion of 18:1 substrate to 18:2, which is the precursor for GLA.

Knutson does not teach how to create an exogenous DHA and/or EPA biosynthetic pathway into an oilseed plant and recovering at least about 1.0:% of

DHA and/or EPA in the seed oil. **This simply was not known prior to Applicants' disclosure.**

Furthermore, it is respectfully submitted that it would not have been obvious to combine the aforementioned references for the reasons set forth above.

Abbott Laboratories, WO 02/02401 discloses elongase genes. This reference does not teach how to create an exogenous DHA and/or EPA biosynthetic pathway into an oilseed plant and recovering at least about 1.0:% of DHA and/or EPA in the seed oil.

Combining references that just disclose enzymes having different substrate specificities does not obviate the instant invention. Knutzon only demonstrated the production of the delta-5 desaturation products of oleic and linoleic acid in seed oil. The disclosure of elongase enzymes in the Abbott Laboratories reference does not teach how to create an exogenous DHA and/or EPA biosynthetic pathway into an oilseed plant and recovering at least about 1.0:% of DHA and/or EPA in the seed oil

Mukerji et al. discloses enzymes involved in the synthesis of polyunsaturated fatty acids. Mukerji et al. do not appear to have done any work in plants. Mukerji et al. does not teach how to create an exogenous DHA and/or EPA biosynthetic pathway into an oilseed plant and recovering at least about 1.0:% of DHA and/or EPA in the seed oil. **This simply was not known prior to Applicants' disclosure.**

Browse et al. just disclose an omega-3 fatty acid desaturase. Again, this does not teach how to create an exogenous DHA and/or EPA biosynthetic pathway into an oilseed plant and recovering at least about 1.0:% of DHA and/or EPA in the seed oil

In view of the foregoing discussion, it is respectfully submitted that the combination of Knutson in view of Abbott Laboratories and further in view of Mukerji et al. or Browse et al. does not teach how to create an exogenous DHA and/or EPA biosynthetic pathway into an oilseed plant and recovering at least about 1.0:% of DHA and/or EPA in the seed oil.

(VIII) Conclusion

In view of the foregoing discussion, it is respectfully submitted that:

a) one of ordinary skill in the art could make and use the claimed invention without engaging in undue experimentation; and

b) claims 1,12, 16 and 26 are not obvious under 35 USC §103(a) over Knutzon et al. (U.S. Patent No. 6, 075, 183 issued June 2000) in view of Abbott Laboratories (WO 02/08401), further in view of each of Mukherji et al. (U.S. 7, 211,656) or Browse et al. (U.S. Patent no. 6,884,921).

Accordingly, the Board is respectfully requested to reverse the final rejection of pending claims 1, 12, 16 and 26 and indicate allowability of all claims.

Enclosed herewith is a Petition for a one (1) month extension of time to permit the filing of the Brief on Appeal. Please charge the fee for extension of time of one (1) month, as well as the requisite fee set forth in 37 CFR §1.17(f), to Appellant's Assignee's (E. I. du Pont de Nemours and Company) Deposit Account No. 04-1928.

Respectfully submitted,

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Dated: February 10, 2009

Claims Appendix

Claim 1. (previously presented) A transgenic oilseed plant that produces mature seeds in which the total seed fatty acid profile comprises at least 1.0% of at least one omega -3 polyunsaturated fatty acid having at least twenty carbon atoms and five or more carbon-carbon double bonds wherein said transgenic oilseed plant comprises in its genome at least two transgenic nucleic acid sequences encoding at least two different polypeptides and further wherein at least one polypeptide has desaturase activity and at least one polypeptide has elongase activity.

Claim 12. (previously presented) The oilseed plant of Claim 1 wherein the polyunsaturated fatty acid is an omega-3 fatty acid selected from the group consisting of eicosapentaenoic acid (EPA), docosapentaenoic acid (DPA), and docosahexaenoic acid (DHA).

Claim 16. (previously presented) Seeds obtained from the plant of Claim 1 wherein said seeds comprise the transgenes.

Claim 26. ((previously presented The plant of Claim 1 or 12 wherein the oilseed plant is selected from the group consisting of soybean, Brassica species, sunflower, maize, cotton, flax, and safflower.

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Molecules of Interest

Eicosapentaenoic acid: biosynthetic routes and the potential for synthesis in transgenic plants

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Accepted 9 October 2003
This review is dedicated to the memory of Dr. David Horrobin (1939–2003).

Abstract

Long chain polyunsaturated fatty acids are now known to play important roles in human health. In particular, eicosapentaenoic acid (20:5$\Delta^{5,8,11,14,17}$; *n*-3: EPA) is implicated as a protective agent in a range of pathologies such as cardiovascular disease and Metabolic Syndrome (Syndrome X). Eicosapentaenoic acid is currently sourced from fish oils, the presence of this fatty acid being due to the dietary piscine consumption of EPA-synthesising micro-algae. The biosynthetic pathway of EPA has been elucidated, and contains several alternative metabolic routes. Progress in using "reverse engineering" to transgenically mobilize the trait(s) for EPA are considered. In particular, the prospect of producing this important polyunsaturated fatty acid in transgenic oilseeds is highlighted, as is the urgent need for a sustainable replacement for diminishing fish stocks.

Keywords: Polyunsaturated fatty acids; Eicosapentaenoic acid; Metabolic engineering; Metabolic Syndrome; Plant biotechnology

1. Introduction

Animal cell membranes primarily consist of a bilayer of phospholipids and cholesterol with imbedded proteins that act as receptors, transporters and enzymes. The phospholipid fatty acid composition determines the physical and functional properties of cell membranes and has important implications for cell integrity and growth, inflammation and immunity. This is defined by the fatty acids esterified to the glycerol backbones, with chain length and unsaturation acting as key determinants (Broun et al., 1999). The role(s) of 20-carbon (C_{20}) polyunsaturated fatty acids (PUFAs) with methylene-interrupted double bonds have been the recent focus of intensive research on fatty acid functionality (Napier et al., 1999, 2003; Gill and Valivety, 1997). For the purpose of this review, PUFAs are defined as containing three or more double bonds on a fatty acid chain of 18 or more carbons. PUFAs can be further classified into two families (*n*-6 or *n*-3), depending on the position of the last double bond proximal to the methyl end of the fatty acid. These *n*-6 and *n*-3 fatty acids (also sometimes called omega-3 and omega-6 fatty acids) are derived from the essential fatty acids (EFA) linoleic acid (LA, 18:2$\Delta^{9,12}$) and α-linolenic acid (ALA, 18:3$\Delta^{9,12,15}$), respectively. Both of these precursor fatty acids are synthesized by plants, but not mammals; therefore they are essential dietary components of all mammals (Groff et al., 1995) and hence their designation as EFAs. Through a series of acyl desaturation and elongation reactions, LA is metabolized to arachidonic acid (AA, 20:4$\Delta^{5,8,11,14}$; *n*-6) and ALA is metabolized to eicosapentanoic acid (EPA, 20:5$\Delta^{5,8,11,14,17}$; *n*-3) and docosahexaenoic acid (DHA, 22:6$\Delta^{4,7,10,13,16,19}$). Thus the *n*-6 (LA) and *n*-3 (ALA) EFAs yield two different distinct classes of C_{20+} PUFAs and these two families are not normally interconvertible. This is highlighted by the fact that they are metabolically and functionally distinct, having opposing physiological functions. PUFAs play key roles in cellular and tissue metabolism, including the regulation of membrane fluidity, electron and oxygen transport, as well as thermal adaptation. They are also implicated in prevention and modulation of certain pathological conditions such as obesity and cardiovascular diseases which now appear common in Western society.

Whilst most mammals have a capacity to synthesise C_{20+} PUFAs from the dietary intake of the precursor

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fatty acids LA and ALA, higher plants in general lack this metabolic pathway. A few taxonomically-unrelated higher plants have the capacity to synthesis Δ^6- and Δ^5-desaturated fatty acids, though it seems unlikely that this is evolutionarily related to mammalian C_{20} PUFA biosynthesis. Another dietary source of $C_{20}+$ PUFAs is delivered by some aquatic micro-organisms which have very active biosynthetic pathways for these lipids. Such marine micro-organisms are consumed by fish and so redistribute the C_{20+} PUFAs up the food-web, culminating in the accumulation of fish oils rich in these fatty acids.

2. PUFAs in human health

2.1. PUFA metabolites

From the medical point of view, the most important function of PUFAs is that they are central to the biosynthesis of a class of compounds termed eicosanoids (i.e. metabolites of eicosa [C_{20}] PUFAs), serving as precursors for these hormone-like regulatory molecules. The ecosanoids consist of prostaglandins (PGs) and thromboxanes (TXs), which are collectively identified as protanoids, and the leukotrienes (LTs). These compounds perform a number of essential physiological functions including regulation of the immune system, blood clotting, neurotransmission and cholesterol metabolism (Funk, 2001). The eicosanoids can be formed when physical or chemical insults result in the release of PUFAs from their phospholipid backbone (through the action of phospholipases) and subsequent oxygenation by local oxygenase enzymes. The type of eicosanoids produced (and hence the body's responses) are determined by multiple factors including: cell-type stimulated (platelets, leucocytes and endothelial cells), oxygenase enzymes present (cyclo-oxygenase versus lipoxygenase) and the actual levels of substrate C_{20} PUFAs in the cell membrane. Eicosanoids derived from *n*-6 fatty acids have different metabolic properties compared to those derived from *n*-3 fatty acids. In general, eicosanoids are classified into several different groups, depending on functionality. For example, series-1 and series-3 which are anti-inflammatory whereas series-2 is pro-inflammatory (see Fig. 1). Eicosanoids derived from the 20:4 *n*-6 AA are generally pro-inflammatory, pro-aggregatory and immuno-active (Hwang, 2000). In contrast, eicosanoids derived from 20:5 *n*-3 EPA have little or no inflammatory activity and act to modulate platelet aggregation and immune-reactivity (Funk, 2001). Thus, there is considerable interest in the "positive" (e.g. anti-inflammatory) effects of *n*-3 (e.g. EPA) derived eicosanoids.

The synthesis of these two families (*n*-6 and *n*-3) of C_{20} PUFAs is mediated by the same enzymes which generally appear to have no particular preference for substrate. Thus, the balance in intake of *n*-6 and *n*-3 fatty acids will therefore determine the types and amounts of eicosanoids in the body and so, influence the strength of the inflammatory response. Consequently, manipulation of the fatty acid composition of cell membranes can theoretically modify the inflammatory, immune and aggregatory responses of tissues, though this understates the complexity of cellular homeostasis. This concept provides the basis for the use of *n*-3 PUFAs as therapeutic agents in the treatment of chronic inflammatory conditions such as rheumatoid arthritis, asthma, psoriasis and Crohn's disease. *N-3* fatty acids may also be involved in the development of non-insulin dependent diabetes as it was shown that a diet low in these fatty acids may favour the development of insulin resistance (Browning, 2003). It is for this reason that dietary intake of *n*-3 PUFAs is considered protective from Metabolic Syndrome (Clarke, 2001; Groop, 2000). Metabolic Syndrome is a multi-component disorder characterised by reduced insulin sensitivity, alterations in circulatory lipids, hypertension and abdominal obesity, conveying an increased risk to cardiovascular disease. Thus, there is considerable interest in the therapeutic role of *n*-3 PUFAs as intervention agents in the prevention and treatment of this disorder.

As mentioned above, the PUFA composition of cell membranes is, to great extent, dependent on dietary intake. The typical Western diet is relatively deficient in *n*-3 fatty acids compared to the diets of our ancestors. Today the ratio of *n*-6–*n*-3 EFAs in modern diets is about 25:1, and when compared with a likely ancestral dietary ratio of <2:1, indicative of a current deficiency in *n*-3 fatty acids (Simopoulos, 1991, 2000). The balance between the intakes of *n*-6 and *n*-3 fatty acids has been suggested to be more important than levels of intake of individual fatty acids for homeostasis and has been postulated to lead to decreases in many chronic diseases and improvement in mental health (Horrobin et al., 2002).

2.2. Omega-3 PUFAs

There are two key *n*-3 fatty acids readily used by the body: EPA and DHA; DHA (decosahexaenoic acid, 22:6, *n*-3) is synthesised directly from EPA as discussed below. These PUFAs are highly concentrated in the brain and appear to be particularly important for cognitive and behavioural function; DHA is also found in retinal cells and is likely to play a key role in the acquisition and maintenance of ocular vision (Uauy et al., 2001). Lipid-lowering effects, along with some benefits in reducing platelet aggregation and clotting potential, make $C_{20}+$ *n*-3 PUFAs very important in the treatment or prevention of cardiovascular disease symptoms (such as high cholesterol and high blood pressure). The decreased blood viscosity and lower fat levels also help reduce the risk of heart attacks. The mild anti-inflammatory effects,

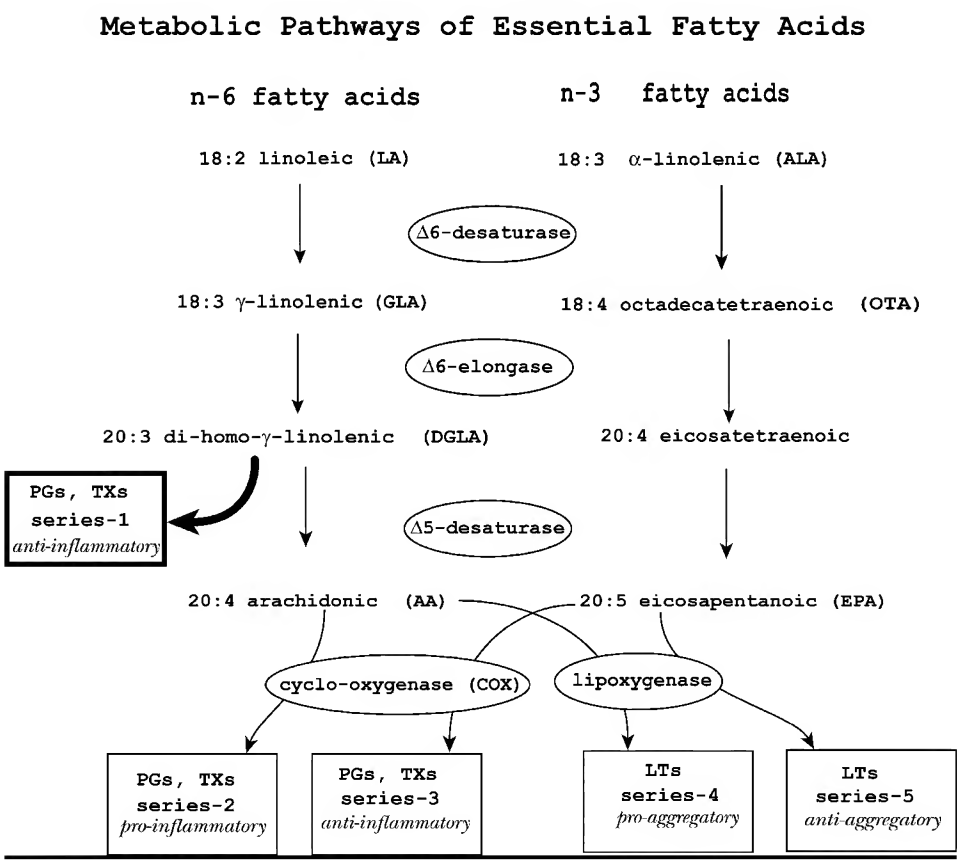

Fig. 1. A simplified scheme of PUFA biosynthesis and eicosanoid metabolism. The two classes (*n*-6, *n*-3) of C_{20} PUFAs are synthesised by desaturation and elongation. Oxygenation of these fatty acids by cyclo-oxygenases and lipoxygenases result in the formation of different types of eicosanoids (prostaglandins, PGs; thromboxanes, TXs; leukotrines, LTs).

possibly a result of increased PG-E1 and PG-E3 prostaglandins, may be helpful in the treatment of arthritis and other inflammatory diseases. In rheumatoid arthritis EPA/DHA supplementation has been shown to reduce joint stiffness and soreness and to improve flexibility. Thrombotic disorders are currently treated and prevented by utilising pharmacological concentrations of EPA (Holman, 1986)

There are also a number of postulated roles for EPA which, whilst of great potential interest, are currently less-well defined. For example, the role of EPA in neurological disorders such as schizophrenia and depression; several studies have shown those with schizophrenia often have low levels of the particular EFAs necessary for normal nerve cell membrane metabolism. Similar to diabetes, people with schizophrenia may not be able to convert efficiently ALA to EPA or DHA (Horrobin, 1999). Studies suggested that patients with schizophrenia and depression experience a sustained clinical improvement in symptoms when given EPA supplements (Edwards et al., 1998; Laugharne et al., 1996), though a recent report questioned the efficacy of PUFAs in schizophrenia (Hibbeln et al., 2003). There are also a number of preliminary studies which link consumption of *n*-3 PUFAs with the development of both infant and adult IQ (Suziki et al., 2001).

At present the only significant direct human dietary sources of EPA and DHA are cold water fish such as cod, tuna and mackerel. However, EPA and DHA occur widely in many unicellular protist species, especially those of marine origin. EPA comprises up to 25% of the total fatty acids of *Eustigmatophytes* and DHA accounts for up to 11% of the total lipids of *Prymnesiophytes* (Brown, 2002). Some fungi, mosses and bacteria also synthesize significant amounts of EPA and DHA; however, higher plants (the major dietary source of fatty acids) rarely contain PUFAs with more than 18 carbon atoms. According to current opinion, *n*-3 PUFAs are synthesised by the microscopic algae and plankton at the bottom of the marine food chain. They are then passed up the food chain (via consumption by omnivorous and subsequently carnivorous fish species), ultimately to humans. However, it has become evident that EPA/DHA from marine sources no longer represents a sustainable resource. In the case of fish (and their derived oils) produced by aquaculture, it is clear that the use of vegetable oil feed rich in *n*-6 C_{18} PUFAs only result in a product rich in *n*-6 fatty acids; this is

clearly not the desired outcome and also generate an EFA-deficiency in the animals. The precipitous decline of European marine fish stocks is well documented, though it is perhaps not appreciated that this not only included primary fish species such as cod, but also so called "trash" species (such as sand eels) which are also utilized (as *n*-3-containing feedstuff) for aquaculture (Sargent and Tacon, 1999). Thus, there is a clear technological push for the development and deployment of a safe, sustainable and cheap alternative source of *n*-3 PUFAs for human health and nutrition. Currently, some microalgal species are cultivated as sources of these fatty acids. However, transgenic oilseed crops engineered to produce EPA and DHA could provide an alternative sustainable source of these important fatty acids (Abbadi et al., 2001). It is for all these reasons that there is great interest in genes encoding enzymes of the PUFA biosynthetic pathway and considerable effort has been expended in their identification. Surprisingly (as outlined below), there is an unexpected diversity in the synthesis of $C_{20}+$ PUFAs by different organisms.

3. Routes for EPA biosynthesis

3.1. Biosynthetic pathways

At present, several alternative pathways for PUFAs biosynthesis resulting in the formation of EPA and DHA have been investigated and genes encoding key enzymatic reaction have been identified (summarised in Fig. 2 and Table 1). The major, or "conventional" aerobic pathway which operates in most (PUFA-synthesising) eukaryotic organisms, starts with Δ^6 desaturation of both 18:2 *n*-6 and 18:3 *n*-3 resulting in the synthesis of γ-linolenic (GLA, 18:3 $\Delta^{6,9,12}$) and octadecatetraenoic (OTA; 18:4 $\Delta^{6,9,12,15}$) acids, respectively. This first desaturation step is followed by Δ^6-specific C_2 elongation to 20:3 $\Delta^{8,11,14}$ and 20:4 $\Delta^{8,11,14,17}$ and further Δ^5-desaturation to produce AA and EPA. From this point, the biosynthesis of DHA may follow two pathways. These are the linear pathway, involving C_2 elongation of EPA to C22:5 $\Delta^{7,10,13,16,19}$ which is desaturated by Δ^4-specific desaturase to yield DHA, and the so-called "Sprecher" pathway which is independent of Δ^4-desaturation but involves two consecutive C_2 elongation cycles to yield 24:5 $\Delta^{7,10,13,16,19}$, followed by Δ^6-desaturation and one cycle of C_2-shorteninig via ß-oxidation in the peroxisome to yield DHA (Sprecher et al., 1995). The existence of the first pathway was confirmed by the isolation of a Δ^4-desaturase from the marine protist *Thraustochytrium* spp. and the freshwater species Euglena (Meyer et al., 2003; Qiu et al., 2001). The second, Δ^4-independent pathway appears more complicated but there is strong experimental evidence that this is the predominant route in mammals (Leonard et al., 2002). Recently, evidence has been presented by several groups that the C_{24} Δ^6-desaturase is the same enzyme as the C_{18} Δ^6-desaturase responsible for the synthesis of GLA and OTA (de Antueno et al., 2001; D'andrea et al., 2002). Also, the completion of the human genome sequence reveals the presence of three cytochrome b_5 fusion PUFA desaturases, two of which have been functionally identified as the Δ^5- and C_{18} Δ^6-desaturases (Cho et al., 1999a,b; Leonard et al., 2000; Marquardt et al., 2000). Whilst it is conceivable that this third cytochrome b_5 fusion sequence encodes a C_{22} Δ^4-desaturase, no evidence has been so far provided to confirm this (or a C_{24}-specific Δ^6-desaturase).

An alternative pathway for the biosynthesis of C_{20} PUFAs has been demonstrated in the protist *Tetrahymena pyroformis*, *Acanthamoeba* spp. and *Euglena gracilis*, organisms which appear to lack Δ^6-desaturase activity (Lees and Korn, 1966: Ulsamer et al., 1969; Wallis and Browse, 1999). The first step in this alternative route is elongation of C_{18} fatty acids, LA and ALA, to eicosadienoic (20:2 $\Delta^{11,14}$) *n*-6 and eicosatrienoic (20:3 $\Delta^{11,14,17}$) *n*-3 fatty acids, respectively. In turn, these C_{20} products are desaturated by a Δ^8-desaturase to produce 20:3 $\Delta^{8,11,14}$ *n*-6 and 20:4 $\Delta^{8,11,14,17}$ *n*-3 PUFAs which are the intermediates of the conventional pathway. The products of Δ^8-desaturation are then subjected to desaturation at the Δ^5 position to produce AA and EPA and may be elongated with subsequent Δ^4-desaturation to DHA. This so called "Δ^8-desaturation" pathway has also been found in rat and human testis (Albert and Coniglio, 1977; Albert et al., 1979) and in glioma and breast cancer cell lines (Cook et al., 1991; Bardon et al., 1996) as well as being hypothesised to explain the synthesis of AA in felines, which appears to lack a Δ^6-desaturase activity (Sinclair et al., 1981). A Δ^8-desaturase has been cloned from Euglena and shown to be structurally related to the other cytochrome-b_5 fusion desaturases (e.g. Δ^6-, Δ^5- and Δ^4-) involved in PUFA synthesis (Watts and Browse, 1999; Napier et al., 2003).

The role of this alternative route of PUFA metabolism in terms of physiological significance in human health remains unclear but this metabolic route for 20:5 *n*-3 formation could be utilised when the conventional route is impaired (either genetically or pathologically). The desaturation catalysed by the Δ^6-desaturase which introduces a double bond at the Δ^6-position of LA and ALA is rate limiting and thus is regulated by dietary factors and hormonal changes. Thus, the alternative pathway may fulfil the task of supplying PUFAs in those tissues where these fatty acids are in greater demand, or not subject to similar regulation. Alternatively, Δ^8-desaturation may take place in mammalian tissues with reduced or zero Δ^6-desaturase activity. Experimental evidence that glioma and breast cancer cells may preferentially elongate 18:3 *n*-3 and that

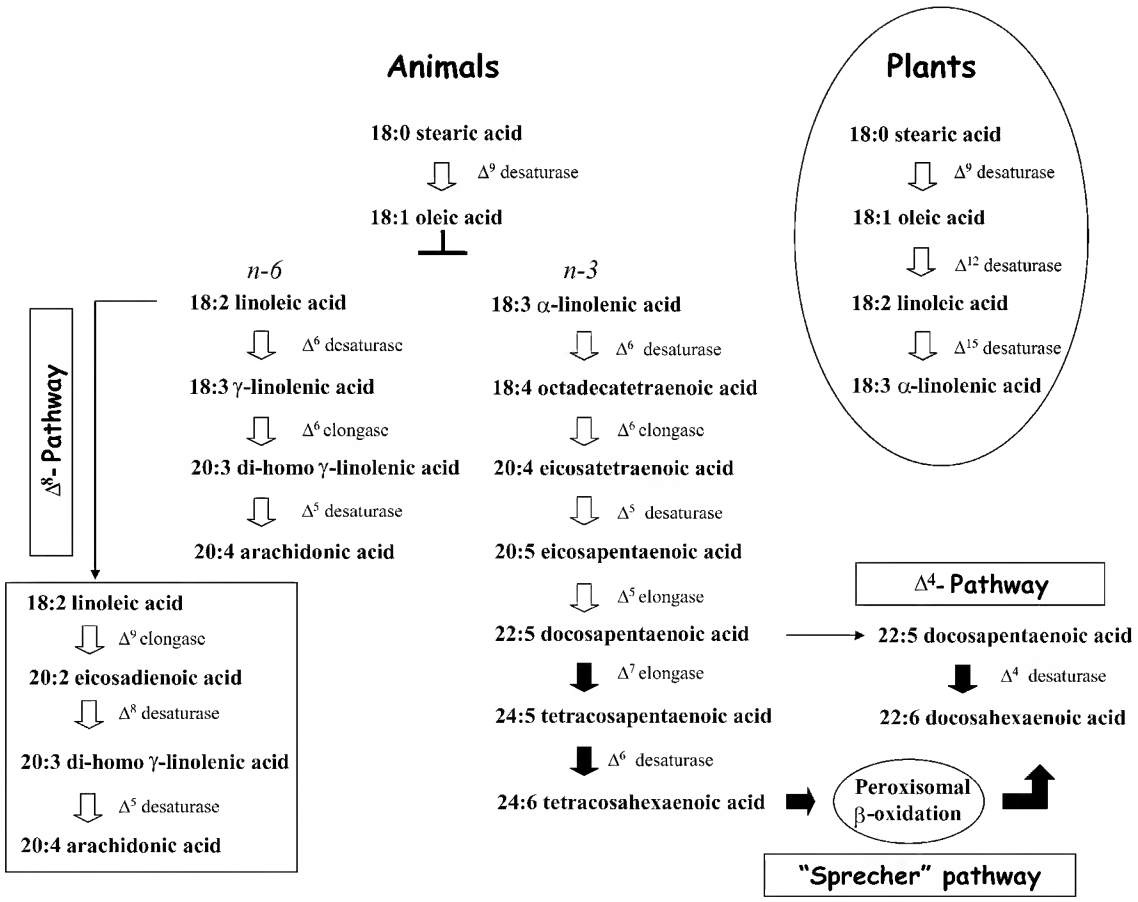

Fig. 2. Biosynthesis of C20+ PUFAs. The various different routes for the aerobic biosynthesis are shown. Precursor EFAs linolenic and a-linolenic acid are the predominant fatty acids synthesised by plants. These then enter the mammalian food web and are subsequently metabolised to C_{20+} PUFAs. The alternative Δ^8-desaturase/Δ^9-elongase pathway is shown, as are the two alternative routes for DHA synthesis. In the case of EPA/DHA synthesising microalgae, C_{20+} PUFAs are synthesised directly from saturated substrates (since such organisms contain both "plant" and "animal" components of the pathway).

Δ^8-desaturation follows this elongation gives support to the hypothesis that Δ^8-desaturation may play an important role in cancerogenesis (Cook et al., 1991; Bardon et al., 1996). However, as mentioned above, the human genome contains only three candidate "front-end" PUFA desaturases, two of which have been functionally characterised. Thus, if the third cytochrome b_5 fusion desaturase is not the C_{22} Δ^4-desaturase, it is conceivable that it encodes a C_{20} Δ^8-desaturase activity. Alternatively, the presence of Δ^8-desaturase activity in cancerous cells could possibly represent a loss of specificity (either directly or indirectly) by the Δ^5- or Δ^6-desaturases. Recently Qi et al. (2002) isolated Δ^9-elongase from C_{22}-PUFA synthesizing microalga *Isochrysis galbana* which had the ability to elongate the C_{18} fatty acids, LA and ALA to 20:2 *n*-6 and 20:3 *n*-3 (i.e. substrates for Δ^8-desaturation). This suggests that the PUFA biosynthetic pathway in *I. galbana* may be similar to that of *Euglena* and utilise the alternative route to provide substrates for Δ^5-desaturation.

In contrast to plants and mammals, some insects and invertebrates such as the nematode *C. elegans* have retained the ability to synthesize C_{20} PUFAs from C_{16-18} saturated and monounsaturated fatty acid, with this latter organism containing all of the enzymes required for the de novo synthesis of *n*-6/*n*-3 C_{20} PUFAs (Hutzell and Krusberg, 1982). The nematode contains Δ^5- and Δ^6-desaturases as well as PUFA elongase activities found in animals and genetic analysis has revealed the importance of PUFA biosynthesis in normal worm development (Napier and Michaelson, 2001; Watts and Browse, 2002; Watts et al., 2003). Moreover, *C. elegans* can perform the crucial step in de novo PUFA biosynthesis by converting 18:1Δ^9 to LA, via a Δ^{12} acyl lipid desaturase (Hutzel and Krasberg, 1982; Tanaka et al 1996); the *C. elegans* gene for this desaturase was recently identified (Peyou-Ndi et al., 2002). This desaturase activity is found in plants but absent in mammals, and its presence (or absence) is likely to represent a key step in the acquisition of dietary requirement for EFAs. *C. elegans* can also convert a range of C_{18} and C_{20} *n*-6 PUFAs into *n*-3 PUFAs through the action of a C_{18-20} *n*-3 desaturase which provides an additional mechanism in the regulation of

Table 1

Enzyme	Type of organism	Species	Refs.
Aerobic desaturases			
Δ^4-desaturase	Algae	*Thraustochytrium sp.*	Qui et al., 2001
		Euglena gracilis	Meyer et al., 2003
Δ5-desaturase	Mammals	*Homo sapiens*	Cho et al., 1999b
	Nematode	*Caenorhabditis elegans*	Michaelson et al., 1998b
	Fungi	*Mortierella alpina*	Michaelson et al., 1998a
		Pythium irregulare	Hong et al., 2002a
	Algae	*Thraustochytrium sp.*	Qui et al., 2001
Δ^6-desaturase	Mammals	*Homo sapiens*	Cho et al., 1999a
		Mus musculus	Cho et al., 1999a
	Nematode	*Caenorhabditis elegans*	Napier et al., 1998
	Plants	*Borago officinales*	Sayanova et al., 1997
		Echium	Garcia-Maroto et al., 2002
		Primula	Sayanova et al., 2003
		Anemone	Whitney et al., 2003
	Mosses	*Ceratodon purpureus*	Sperling et al., 2000
		Physcomitrella patens	Girke et al., 1998
	Fungi	*Mortierella alpina*	Chaudhary et al., 1999
		Pythium irregulare	Hong et al., 2002b
Bifunctional Δ^5/Δ^6 desaturase	Fish	*Danio rerio*	Hastings et al., 2001
C_{20} Δ^8-desaturase	Protist (green algae)	*Euglena gracilis*	Wallis and Browse, 1999
C_{18-20} *n*-3 desaturase	Nematode	*Caenorhabditis elegans*	Spychalla et al., 1997
Aerobic elongases			
Δ^6-elongases	Nematode	*Caenorhabditis elegans*	Beaudoin et al., 2000
	Mosses	*Physcomitrella patens*	Zank et al., 2000
	Fungi	*Mortierella alpina*	Parker-Barnes et al., 2000
PUFA-elongase	Mammals	*Homo sapiens*	Leonard et al., 2002
Δ^9-elongase	Algae	*Isochrysis galbana*	Qi et al., 2002
Anaerobic enyzmes			
EPA-polyketide synthase	Bacteria	*Shewanella putrefaciens*	Takeyama et al., 1997
DHA-polyketide synthase	Algae	*Schizochytrium* sp.	Metz et al., 2001
	Bacteria	*Moritella marinus*	Metz et al., 2001

n-3 PUFAs supply. Thus, the ratio of *n*-3/*n*-6 PUFAs is higher in *C. elegans* than in mammals because the presence of the (additional) *n*-3 desaturase activity makes the two parallel *n*-6/*n*-3 PUFA biosynthetic pathways more independent from the dietary intake of *n*-6 and *n*-3 fatty acids.

Functional characterization of cDNA clones corresponding to *C. elegans* Δ^5-, Δ^6-, Δ^{12}-(designated FAT-3, FAT-4 and FAT-2, respectively) and the C_{18-20} *n*-3 (designated FAT-1) desaturase genes have confirmed their enzymatic activities (Napier et al., 1998; Michaelson et al., 1998a,b; Watts and Browse, 1999; Spychalla et al., 1997; Peyou-Ndi et al., 2002). One intriguing aspect of *C. elegans* PUFA biosynthetic pathway is the obvious evidence of gene duplication; this is true for both the related FAT-1/FAT-2 genes and the Δ^5- and Δ^6-desaturases. This may serve as a paradigm for the evolution of distinct enzyme activities from an ancestral prototypic (bifunctional) enzyme (Napier and Michaelson, 2001). In that respect, the identification of a bi-functional Δ^6-/Δ^5-desaturase from zebrafish may represent such an archetype (Hastings et al., 2001).

Whilst most flowering plants have no capacity to synthesis C_{20} PUFAs, some other (lower) organisms can synthesize PUFAs from saturated fatty acids. The moss *Physcomitrella patens* synthesises EPA via the conventional *n*-3 PUFAs pathway involving Δ^6/Δ^5-desaturation and elongation steps, as witnessed by the isolation of genes encoding these activities (Girke et al., 1998; Zank et al., 2000). The fungus *Mortierella alpina* and many species of algae possess all the enzyme activities to synthesize EPA from saturated substrates (Parker-Barnes et al., 2000; Chaudhary et al., 1999). Studies of PUFAs biosynthesis with radiolabeled precursors have shown that *M. alpina*, *Porphyridium cruentum*, *Crypthecodinium cohnii* and *Nannochloropsis* use a similar pathway leading to EPA biosynthesis (Khozin et al., 1997; Henderson and Mackinlay, 1991; Schneider and Roessler, 1994

Recently a very different and much simpler anaerobic biosynthetic pathway has been identified for both prokaryotic and eukaryotic marine organisms (Metz et al., 2001; Napier, 2002). It is catalysed by a multifunctional complex that is analogous to polyketide synthases (PKS) and does not require PUFA-specific desaturases and elongases. Several PKS gene clusters from C_{20}-PUFA accumulating marine bacteria *Shewanella* and *Vibrio marinus* were expressed in *E. coli* and *Synechoccus* result-

ing in the accumulation of EPA and DHA (Takeyama et al., 1997; Tanake et al., 1996). The identification of a PKS-like pathway in a marine protist *Schizochytrium*, a member of the *Thraustochytriaceae*, indicates that it can be widespread in marine ecosystem (Metz et al., 2001). It also raises a question as to the evolutionary relevance of the aerobic desaturase/elongase pathway present in *Thraustochytrium* (another member of the *Thraustochytriaceae*) (Qiu et al., 2001; Napier, 2002).

3.2. Identification of genes involved in n-3 PUFA biosynthesis

During last few years most of the genes responsible for the biosynthesis of the *n*-3 PUFAs have been cloned from various organisms including algae, fungi, mosses, plants and mammals (summarised in Table 1). The desaturation enzymes required for PUFA biosynthesis are membrane-bound proteins which have three strongly conserved histidine-rich sequences (His boxes) comprising the general motifs H–$X_{[3-4]}$H, H–$X_{[2-3]}$H–H and H/Q–$X_{[2-3]}$H–H (Shanklin et al., 1994; Shanklin and Cahoon, 1998; Sperling et al., 2003). Based on regioselectivity these desaturases can be separated into two different groups: the "methyl-end" desaturase introducing the next double bond between the existing one and the methyl end of the fatty acid chain, and the "front-end" desaturases, inserting a new double bond between an existing one and the carboxyl end of the acyl group in a methylene-interrupted pattern (Somerville and Browse, 1996; Napier et al., 1997). All current examples of "front end" desaturases (introducing Δ^6-, Δ^5-, Δ^4- and Δ^8-double bonds) contain a N-terminal cytochrome b_5 domain which serves as the electron donor for desaturation (Napier et al., 1997, 1999, 2003; Sperling et al., 2003). Another characteristic of the "front end" desaturases is the substitution of histidine by glutamine in the third histidine box. Site-directed mutagenesis of this glutamine to (a consensual) histidine resulted in loss of the desaturase activity; thus, the consensus sequence for the third His box of front-end desaturases is more correctly given as Q–$X_{[2-3]}$–H–H (Sayanova et al., 2001).

The first example of the gene encoding the key reaction in both *n*-6 and *n*-3 metabolic pathways, the microsomal Δ^6-fatty acid desaturase, was cloned from borage (*Borago officinalis*) and expressed in tobacco plants and in yeast (Sayanova et al., 1997, 1999). These studies demonstrated the formation of GLA and OTA in transgenic plants and also in yeast (in the presence of exogenous substrates, LA and ALA). Orthologs of the Δ^6-desaturase have been identified from many different species including *C. elegans* (Napier et al., 1998; Watts and Browse, 1999), mammals (Cho et al., 1999a,b, fungi (Huang et al., 1999; Hong et al., 2002a,b), mosses (Girke et al., 1998; Sperling et al., 2000) and plants (Garcia-Maroto et al., 2002; Whitney et al., 2003). All these Δ^6-desaturases showed no major preference for *n*-6 or *n*-3 substrates (Browse et al.,1999; Huang et al., 1999; Girke et al., 1998; Sperling et al., 2000), though recently we have cloned and characterized two members of Δ^6-fatty acid desaturases from *Primula* with strong specificity towards *n*-3 substrates (Sayanova et al., 2003). As mentioned above, the presence of 24:6 *n*-3 metabolites in mammalian cells supported the hypothesis of the existence of two distinct Δ^6-desaturase activities specific to chain length (C_{18} v. C_{24}). Until recently, there was little conclusive evidence that the Δ^6-desaturase which acts on the C_{18} unsaturated fatty acids is also capable of desaturating C_{24} substrates. Two recent studies have demonstrated that both human and rat single Δ^6-desaturases act on C_{18} and C_{24} PUFAs (de Antueno et al., 2001; d'Andrea et al., 2002). Interestingly, the rat Δ^6-desaturase is more active on C_{18} *n*-3 than C_{24} *n*-3 (d'Andrea et al., 2002). However, these results do not exclude the possibility of the existence of another (substrate-specific) Δ^6-desaturase activities (or even a Δ^4-desaturase) in other mammals.

The second key enzyme in the *n*-3 PUFAs pathway is the Δ^5-desaturase that catalyses the last desaturation step of EPA biosynthesis from 20:4 *n*-3 and sequences encoding this activity have been identified from various organisms (Table 1). The cloned Δ^5-desaturases were expressed in yeast in the presence of exogenous substrates and demonstrated their ability to produce Δ^5-unsaturated fatty acids. The Δ^5-desaturases cloned from *M. alpina* and *C. elegans*, when expressed in heterologous systems, acted on a range of substrates, inserting double bonds in a non-methylene-interrupted pattern. Expression of the *M. alpina* Δ^5-desaturase in transgenic canola resulted in the production of 18:2 $\Delta^{5,9}$ (taxoleic acid) and C18:3 $\Delta^{5,9,12}$ (pinolenic acid) (Knutzon et al., 1998), whereas the nematode enzyme (when expressed in yeast in the presence of exogenous substrates) produced 20:3 $\Delta^{5,11,14}$ and 20:4 $\Delta^{5,11,14,17}$ in addition to endogenously produced 18:2 $\Delta^{5,9}$ (Watts and Browse, 1999). The presence of two Δ^5-desaturases from the slime mould *Dictyostelium discoideum* have also been reported to produce taxoleic acid and 18:2 $\Delta^{5,11}$ with one of these enzymes able to act on the saturated substrate, 16:0 (Saito and Ochiai, 1999). At present there is no experimental evidence for the use of C_{20} PUFAs as the substrates for *Dictyostelium discoideum* Δ^5-desaturases. However, small amounts of 20:3 $\Delta^{5,11,14}$ and 20:4 $\Delta^{5,11,14,17}$ have been identified in several species of slime molds (Rezanka, 1993) which leaves the possibility that these two Δ^5-desaturases can act on the C_{20} conventional Δ^5 substrates.

As already described, the Δ^5-desaturation of eicosatetraenoic acid (20:4 *n*-3) is the last step on the biosynthetic pathway of EPA. The previous step, in which 20:4 *n*-3 is synthesized from OTA, is catalyzed by a C_2

elongase complex. Biochemical evidence suggests that the fatty acid elongation consists of four steps: condensation, reduction, dehydration and a second reduction (Cinti et al., 1992). At present, identified condensing enzymes (3-keto-acyl synthases) can be divided into two groups. A first group includes the FAE-like plant enzymes involved in the biosynthesis of saturated and monounsaturated fatty acids with C_{18-22} chain length, with these enzymes showing homology to other condensing enzymes such as chalcone synthase (Millar and Kunst, 1997); so far, there is no evidence that this FAE1-like class of enzymes is involved in the synthesis of PUFAs. However, a second class of (presumptive) condensing enzymes has been defined by the ELO gene family of yeast, which are required for the synthesis of VLCFA components of sphingolipids (Oh et al., 1997). Apparent paralogs of the ELO-type class of (sphingolipid) VLCFA elongases have recently been demonstrated to be involved in PUFA biosynthesis. For example, several C_{18} Δ^6-specific elongases from fungus, moss and nematode have been cloned and characterized by heterologous expression in yeast (Beaudoin et al., 2000; Parker-Barnes et al., 2000; Zank et al., 2000), whilst a related C_{18} Δ^9-elongating activity of the alternative Δ^8-desaturation pathway has also recently been identified from *Isochrysis* (Qi et al., 2002). The molecular basis for this substrate recognition (Δ^6- versus Δ^9-C_{18} PUFAs) is currently undefined, as is the precise enzymatic function of these ELO-like ORFs, though as mentioned above, they are assumed to be condensing enzymes.

4. Biotechnology of PUFA production

The existence of different pathways for PUFAs biosynthesis offers a wide range of alternatives to budding plant biotechnologists in their quest to produce desired fatty acids in transgenic oilseed crops. At present, the most obvious approach is based on the use of aerobic "front-end" desaturases and elongases. The C_{20} PUFA biosynthetic pathway has been successfully reconstituted in yeast by the co-expression of the Δ^6-elongase with Δ^6- and Δ^5-fatty acid desaturases resulted in small but significant accumulation of AA and EPA from exogenously supplied LA and AL (Beaudoin et al., 2000; Zank et al., 2000)

The first step towards engineering oilseeds to produce PUFAs such as EPA was expression of the borage Δ^6-fatty acid desaturase in transgenic tobacco and *Arabidopsis* plants (Sayanova et al., 1997, 1999). Data obtained from these experiments indicated that the (constitutive) expression of borage Δ^6 desaturase in transgenic plants resulted in the production of 18:3 *n*-6 and 18:4 *n*-3 which clearly indicated that the unusual fatty acids required for PUFAs biosynthesis can be incorporated into glycerolipids of the host plants (unlike other non-native fatty acids in transgenic plants; Suh et al., 2002). A step forward in the assembling of the PUFAs pathway in oilseeds was the co-expression of the *M. alpina* Δ^6- and Δ^{12}-desaturases in canola plant resulted in the accumulation of up to ~50% of GLA (Huang et al., 2001). More recently, we have obtained transgenic soybeans expressing the borage Δ^6-desaturase under the control of a seed-specific promoter, resulting in the accumulation of GLA to almost 50% of seed fatty acids (A.J. Kinney, personal communication). These data indicate that it is very likely that GLA is not restricted to a single position (such as sn-2) on the glycerolipids backbone. Since accumulation of high levels of GLA is a prerequisite for the successful reconstitution of the C_{20} PUFA biosynthetic pathway (Fig. 2), these results bode well for future experiments. Currently, considerable effort is focussing on the combined expression in plants of the Δ^6-desaturase with the Δ^6-elongase and the Δ^5-desaturase; publication of these data is eagerly awaited. However, since the "reverse engineering" of any PUFA biosynthetic pathway requires the transgenic mobilisation of multiple different enzyme activities, this will require the heterologous expression of a minimum of three transgenes. The different reverse engineering strategies for the synthesis of C_{20+} PUFAs in transgenic plants are summarized below.

4.1. Routes for C_{20+} PUFA synthesis

Aerobic	
Synthesis of EPA	
• Conventional aerobic desaturation	
Δ^6-desaturase, Δ^6-elongase, Δ^5-desaturase	Yields AA, EPA
• Alternative aerobic desaturation	
Δ^9-elongase, Δ^8-desaturase, Δ^5-desaturase	Yields AA, EPA
Additional pathway "skews"	
• *N-3*-specific Δ^6-desaturase	Yields OTA
• C_{18-20} w-3 desaturase	Yields OTA, EPA
Conversion of EPA to DHA	
• Convention aerobic desaturation	
Δ^5-elongase, Δ^4-desaturase	Yields DPA, DHA
• "Sprecher" peroxisomal shunt	
Δ^5-elongase, Δ^7-elongase, Δ^6-desaturase (requires 1 cycle of β-oxidation to convert THA to DHA)	Yields THA, DHA
Anaerobic	
Polyketide synthase-like processive synthesis	Yields EPA/DHA

At present, the availability of PUFA elongases and desaturases makes real the possibility of producing a wide range of PUFAs in oilseed crops. However, this is likely to be sub-optimal in efficiency when compared with the original gene sources, as has been observed in other attempts to engineer (more simple) fatty acid traits in transgenic plants (Drexler et al., 2003; Suh et al., 2002). This could be due to multiple factors, such as those resulting from the synthesis of non-native fatty acids, variations in substrates used for desaturation (glycerolipid versus acyl-CoA) (Domergue et al., 2003), as well as the random nature of transgenes integration into plant genomes. Another consideration is the promiscuous behaviour of enzymes in heterologous systems, resulting in activities towards either new or unexpected substrates. For example, the expression of the *M. alpina* Δ^5-desaturase in transgenic canola resulted in the accumulation of C_{18} Δ^5-desaturated fatty acids, even though these products are not detected in the endogenous lipids of the fungus (Knutzon et al., 1998). Therefore, the selection of genes encoding enzymes with high chain-length selectivity becomes a particularly important issue. Whilst Δ^6- or Δ^5-unsaturated metabolites as 16:1Δ^6 or taxoleic, pinolenic acids and C20:2 $\Delta^{5,11}$ are not considered to be normal intermediates in C_{20} PUFA biosynthesis, their physiological effects (as minor compounds in a transgenic plant) would still certainly need to be considered from human nutritional and biochemical persectives. Therefore, an additional target for biotechnological applications would be the identification of high-fidelity enzymes for production of PUFAs without the formation of unintended by-products resulting from substrate promiscuity.

5. Future prospects

Whilst the synthesis of EPA requires only three enzyme activities (Table 1), the longer term objective might be the synthesis of DHA. Since this C_{22} PUFA is synthesised by additional elongation and desaturation of EPA, synthesis in a heterologous host will require additional enzyme activities (and hence, transgenes). Initial attempts to heterologously reconstitute DHA synthesis in yeast appear to have demonstrated low but significant levels of this PUFA, marking an important "proof-of-concept" A. Abbadi and E. Heinz, oral presentation. It remains to be seen if this (or other) aerobic desaturase/elongase pathways are the best system for the transgenic synthesis of C_{20+} PUFAs, or if the anaerobic PKS-like system is a viable alternative.

Whatever routes are used for the transgenic synthesis of C_{20+} PUFAs such as EPA, it is clear that there is an urgent need for alternative and sustainable sources of these fatty acids (Sargent and Tacon, 1999). However, it is equally clear that continued consumer antipathy (exacerbated by co-ordinated lobbying from unelected pressure groups) currently limits the possibility for deploying transgenic plants nutritionally-enhanced with C_{20} PUFAs into the human food chain. One alternative for the provision of the health-beneficial PUFAs such as EPA sourced from transgenic plants would be to use them as feedstuffs in commercial aquaculture. As described above, fish accumulate C_{20+} PUFAs primarily as a result of dietary intake of EPA and DHA-rich micro-organisms, and not as a result of endogenous biosynthetic capacity. This translates into an absolute requirement for EPA/DHA in the supplements used to feed fish under aquaculture. Thus, it is possible to envisage a situation in which EPA-enriched oils, derived from transgenic plants, are used to replace current (less sustainable) "trash" fish sources of these PUFAs in aquaculture. In this way, the significant health benefits of these fatty acids could be delivered into the human diet, without the requirement for the direct ingestion of GM food.

6. Conclusions

Virtually all the genes encoding the enzyme activities required for primary C_{20+} PUFA biosynthesis have now been cloned and functionally characterised, and the possibility of heterologous reconstitution of this pathway has been demonstrated in yeast. The first steps towards the "reverse engineering" of EPA synthesis in transgenic plants look extremely promising and it is to be anticipated that further advances (via the introduction of additional PUFA synthesising activities) will be made. The use of transgenic plants to synthesise fish oils may not only provide a sustainable source of these important fatty acids, but may also help demonstrate the utility of GM technology to enhance human health and nutrition.

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

IN THE APPLICATION OF:

ANTHONY J. KINNEY ET AL. CASE NO.: BB1538USNA

SERIAL NO.: 10/776311 GROUP ART UNIT: 1638

FILED: FEBRUARY 11, 2004 EXAMINER: FOX, DAVID T.

FOR: PRODUCTION OF VERY LONG CHAIN POLYUNSATURATED FATTY ACIDS IN OIL SEED PLANTS

Assistant Commissioner for Patents
Washington, DC 20231

Sir:

Declaration of Dr. Anthony John Kinney Pursuant to 37 CFR 1.132

I, Anthony John Kinney do hereby declare as follows:

1. I am a citizen of the United Kingdom and am a permanent resident of the United States of America, residing at 609 Lore Avenue, Wilmington, Delaware 19809.

2. I received a B. Sc. in biology from the University of Sussex in 1980 and a D. Phil. in biochemistry and cell biology from Oxford University in 1985.

3. I served as a research fellow in the Department of Food Science at Rutgers University, New Brunswick, N.J. 9/87-5/89.

4. I have been employed at E. I. du Pont de Nemours and Company (DuPont) since June 1989 and presently work as a principal investigator for DuPont's agricultural products and am presently working on expression of storage oil genes.

5. I have authored in excess of fifteen refereed articles in the field of biochemistry.

6. I have reviewed the above-identified case, and the Official Action for the subject case dated January 25, 2007. I understand that this declaration is being submitted to address the rejections of the pending claims

under 35 USC §112, first paragraph, and the concerns raised during the interview held on February 13, 2007. Specifically, this declaration is intended to demonstrate that there are a variety of genes (from various sources) and combinations thereof that can be used to engineer the production of omega-3 fatty acids in oilseed crops.

It should be noted at the outset that the claimed invention really constitutes a pioneering invention which has provided a foundation to help other researchers to engineer the production of omega-3 fatty acids in oilseed crops. Prior to this disclosure, the possibility that one could produce long chain omega-3 fatty acids in the oil fraction of seeds was not known.

The references discussed below all refer to and cite the ground-breaking work that is disclosed and claimed in the above-identified application. I truly believe that accomplishments achieved in my laboratory in this area are at the forefront of work in this field as evidenced by the extent to which others have relied and continue to rely on research foundation that we have provided.

Submitted herewith is a copy of an article co-authored by the undersigned and Dr. Howard G. Damude who are also co-inventors of the above-identified application. This article was published recently in Lipids on or about March 14, 2007 and is entitled "Engineering oilseed plants for a sustainable, land-based source of long chain polyunsaturated fatty acids."

Attention is kindly invited to the bottom of page3, second column of the article specifically, the section labeled "Engineering omega-3 LCPUFA into Plants: Fatty Acid Biosynthetic Pathways." This section presents a nice overview of information available to those skilled in the art. It is stated on page 8 that:

> In contrast to PKS synthases, the pathways of aerobic ARA, EPA and DHA synthesis use a series of individual desaturase and elongase activities to catalyze the conversion of LNA and ALA to LCPUFA (34). For ARA and EPA synthesis this requires the addition of 2 carbons and two double-bonds to LNA and ALA respectively. ARA can be converted to EPA by the action of a third (omega-3) desaturase (35).
>
> Two converging ARA/EPA pathways have been identified in LCPUFA-producing organisms (*Figure 1*); in both pathway types LNA and ALA are the metabolic precursors. In the first pathway type, LNA and ALA are first desaturated to gamma-linolenic acid [GLA, 18:3(6,9,12)] and stearidonic acid [STA, 18:4(6,9,12,15)], respectively, by a delta-6 fatty acid desaturase. These fatty acids are then elongated

> to 20-carbons by a microsomal fatty acid elongation complex (28). This elongation is initiated by a delta-6 specific beta-ketoacyl-CoA synthase enzyme (delta-6 elongase). The 20-carbon ketoacyl-CoA is then reduced, dehydrated and reduced again by the elongation complex to yield dihomo-gamma-linolenic acid [DGLA, 20:3(8,11,14)] or eicosatetraenoic acid [ETA, 20:4(8,11,14,17)]. These fatty acids are then desaturated to ARA and EPA respectively by a delta-5 desaturase.
>
> In the second pathway type, LNA and ALA are first elongated by a delta-9-specific elongase to eicosadienoic acid [EDA, 20:2(11,14)] and eicosatrienoic acid [ERA, 20:3(11,14,17)], followed by delta-8 desaturation to DGLA and ETA, respectively. As in the first pathway, these fatty acids are then desaturated to ARA and EPA respectively by a delta-5 desaturase.
>
> Independent of the aerobic pathway utilized, some organisms have the added capability of efficiently converting omega-6 fatty acids to omega-3 fatty acids by the action of an omega-3 fatty acid desaturase (35-39). This desaturation can occur on either 18-carbon or 20-carbon fatty acids. . . .

It is noted in this article on page 4, column 2, that the soybean study, described in WO 2004/071467 which is the PCT equivalent of the above-identified application, involved characterization of multiple seed-specific promoters and LCPUFA synthetic genes from different microbial sources in addition to optimization of promoter-gene cassette combinations and orientations in soy.

Several references were discussed at the above-referenced February 13 interview.

Robert et al. is one of the references that was discussed during the above-identified interview held on February 23, 2007 and in the last Office Action and previously filed response.

Attention is kindly invited to Table 1 appearing on page 105 of Robert et al. This table presents a summary of genes, host plants and reported LCPUFA proportions in seeds of transgenic plants.

Another reference that was discussed is Napier et al., Physiologia Plantarum 126:398-406 (2006) which is a review of plant metabolic engineering of very long-chain polyunsaturated fatty acids in transgenic plants. The research that I and my group conducted and which constitutes the subject matter of the above-identified application is discussed on page 402, second column through the first column on page 403. The work of Roberts et al. and Wu et al. is also discussed. This review shows that a

variety of approaches using different genes and combinations thereof to make transgenic plants producing very long-chain polyunsaturated fatty acids.

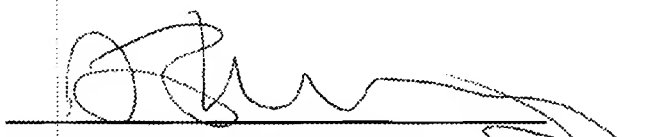
One other reference that was discussed at this interview was Wu et al., Nature Biotechnology 23(8):1013-1017 (August 2005). This is the same work mentioned by Roberts et al. and Napier et al. Wu et al. discussed the transgenic production of arachidonic acid in Brassica juncea seeds.

Parenthetically, a question was raised at the interview regarding sources of a delta-17 desaturase other than Saprolgenia diclina. A publication from Spychalla et al. PNAS 94:1142-1147 (1997) describing a delta-17 from C. elegans was mentioned during the conversation. A copy of this reference is attached hereto.

Attention is also kindly invited to Wu et al. which describes the use of an omega-3 desaturase (D17 desaturase) from Phytophtora infestans (Acc# CS160901) and EPA increases from average of 1.4% to 8.1% with concurrent decrease in ARA (also WO 2005/083093)

It is respectfully submitted that in view of the foregoing, it should be clear that there are variety of genes and combinations thereof can be utilized to engineer oilseed crops to produce LCPUFAs in oilseed plants. This observation is further depicted in Figure 1 of the above-identified pre-publication.

I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.



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INVITED THEMATIC REVIEW

Engineering Oilseed Plants for a Sustainable, Land-Based Source of Long Chain Polyunsaturated Fatty Acids

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Abstract Numerous clinical studies have demonstrated the cardiovascular and mental health benefits of including very long chain omega-3 polyunsaturated fatty acids, namely eicospentaenoic acid (EPA) and docosohexaenoic acid (DHA) in the human diet. Certain fish oils can be a rich source of omega-3 long chain polyunsaturated fatty acids although processed marine oils are generally undesirable as food ingredients because of the associated objectionable flavors and contaminants that are difficult and cost-prohibitive to remove. Oilseed plants rich in omega-3 fatty acids, such as flax and walnut oils, contain only the 18-carbon omega-3 polyunsaturated fatty acid alpha-linolenic acid, which is poorly converted by the human body to EPA and DHA. It is now possible to engineer common omega-6 rich oilseeds such as soybean and canola to produce EPA and DHA and this has been the focus of a number of academic and industrial research groups. Recent advances and future prospects in the production of EPA and DHA in oilseed crops are discussed here.

Abbreviations

LCPUFA	Long chain polyunsaturated fatty acids
DHA	Docosahexaenoic acid
EPA	Eicosapentaenoic acid
ARA	Arachidonic acid
COX-2	Cyclooxygenase-2
LNA	Linoleic acid
ALA	Alpha-linolenic acid
GLA	Gamma-linolenic acid
STA	Stearidonic acid
DGLA	Dihomo-gamma-linolenic acid
ETA	Eicosatetraenoic acid
EDA	Eicosadienoic acid
ERA	Eicosatrienoic acid
DPA	Docosapentaenoic acid
CoA	Coenzyme A
PtdCho	Phosphatidylcholine
LPAAT	Lysophosphatidic acid acyltransferase
SCA	Sciadonic acid
JUN	Juniperonic acid
PKS	Polyketide synthase

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Manipulating the Fatty Acid Content of the Human Diet

The main sources of oils and fats in the human diet are oilseed crop plants, mostly soy, canola (oilseed rape), palm, peanut and sunflower. Many of the oils from these crops are rich in 18-carbon omega-6 fatty acids. It has been demonstrated that excess consumption of omega-6 fatty acids leads to the depletion of omega-3 fatty acids in human body tissues, with numerous negative health consequences [1]. Hydrogenated or partially hydrogenated vegetable oils also contribute to the sensory characteristics of numerous processed food products. Edible vegetable oils are hydrogenated to improve shelf-life, maintain the flavor and provide the expected mouth-feel and consistency of oil-containing foods [2]. Hydrogenation leads to the formation of *trans* unsaturated fatty acids, which provide the

necessary solid-fat functionality for certain food applications. However, the negative health consequences of *trans* unsaturated fatty acid consumption on human health has become better understood in recent years [3].

The first successful attempts of genetically manipulating the fatty acid profile of omega-6 oilseed crops were focused on the redirection of fatty acid biosynthesis in the developing seed, either by blocking specific steps, such as fatty acid desaturation [4], or introducing single enzyme activities to redirect fatty acid synthesis to new end products that provided the required functionality [5]. By these means it was possible to improve the oxidative stability and provide a solid fat functionality in vegetable oils without the need for hydrogenation and the consequent formation of *trans* fatty acids [6, 7].

With advances in gene expression technology it is now possible to consider more complex manipulations of plant cell lipid metabolism, such as the introduction of entire metabolic pathways. Thus it is theoretically possible, for example, to transfer a metabolic pathway for EPA or DHA synthesis from a marine organism to an oilseed crop plant. This would provide an abundant, clean, sustainable and relatively inexpensive source of omega-3 long chain polyunsaturated fatty acids for the human diet. Here we discuss metabolic engineering efforts to achieve this goal.

Long Chain Polyunsaturated Fatty Acids Human Health

Long chain polyunsaturated fatty acids (LCPUFA) are important components of cell membrane phospholipids in humans. Docosahexaenoic acid (DHA, 22:6 [4, 7, 10, 13, 16, 19]), for example, is an important component of mammalian retinal and brain membranes and has been shown to play a role in the cognitive development of infants as well as the mental health of adults [8, 9, 10]. Numerous studies [11–14] have shown cardiovascular health benefits arising from the consumption of eicosapentaenoic acid (EPA, 20:5 [5, 8, 11, 14, 17]). LCPUFA are also precursors to the eicosanoid family of metabolites which include prostaglandins, leukotrienes, thromboxanes [15, 16]. These molecules regulate certain key metabolic functions in the human body, such as inflammatory responses and the induction of blood clotting as well as the regulation of blood pressure [17]. Eicosanoids derived from omega-6 LCPUFA, such as arachidonic acid (ARA, 20:4 [5, 8, 11, 14]), are generally pro-inflammatory while those derived from omega-3 LCPUFA, such as eicosapentaenoic acid, are anti-inflammatory [12, 16, 18].

The first step in eicosanoid biosynthesis from LCPUFA is catalyzed by the cyclooxygenase-2 (COX-2) enzyme [16], which can utilize either ARA or EPA as a substrate. Thus, in addition to the anti-inflammatory action of omega-3-derived eicosanoids themselves, the anti-inflammatory action of EPA can be attributed to its competitive inhibition of ARA for COX-2 [18]. Thus an optimal balance of omega-3 and omega-6 LCPUFA must be achieved to maintain a healthy state.

Direct inhibition of COX-2 activity is the mechanistic basis of a whole class of non-steroidal, anti-inflammatory pharmaceuticals used in the treatment of arthritis and similar conditions. However, while EPA and DHA are beneficial to cardiovascular health, preventing their conversion to eicosanoids by anti-arthritis pharmaceuticals can result in various negative cardiovascular side effects. Indeed negative cardiovascular side effects have been attributed to the use of some COX-2 selective inhibitors in some individuals [19]. It is apparent, therefore, that achieving a balanced dietary intake of omega-6 and omega-3 fatty acids is the most preferred means of preventing negative inflammation responses and of maintaining cardiovascular health for large segments of the population.

Eicosapentaenoic acid and ARA can be synthesized in the human body from the essential dietary fatty acids linoleic acid (LNA, 18:2 [9, 12]) or alpha-linolenic acid (ALA, 18:3 [9, 12, 15]), respectively [20]. The conversion of LNA and ALA to ARA and EPA is relatively inefficient and EPA, DHA and ARA can also be obtained more efficiently directly from the diet, mainly from the consumption of fish and fish-oil [16, 20]. In Western societies, the dietary ratio of omega-6 to omega-3 fatty acids has shifted heavily toward omega-6 fatty acids over the past 60 years [21]. This shift is the result of an overall decrease in the consumption of fish and fish oils, which contain high levels of omega-3 LCPUFA, as well as a large increase in the consumption of omega-6-containing foods, such as common vegetable oils or grain-fed meat and poultry [21]. By some estimate the current omega-6 to omega-3 intake in the human diet is as much as 30-fold too high [21, 23]. This has led to a general imbalance of ARA and EPA in the blood stream with numerous possible negative consequences [21]. Thus consumption of foods rich in omega-3 LCPUFA may help to correct this imbalance by shifting the omega-6 to omega-3 fatty acid ratio to more optimal levels in the human body.

An increasing demand for fish and fish oils high in omega-3 LCPUFA is putting an even greater stress on an already overexploited resource [24]. In addition, the cost associated with removing objectionable odors and flavors, as well as contaminants such as mercury and PCBs, generally limits the use of fish oils as food ingredients [25, 26]. Microalgal-derived omega-3 oils produced through fermentation are free from the contaminants found in fish oils but their high cost restricts their use to infant formula and medical foods and generally prohibit their inclusion in common food products.

A potentially cost-effective and sustainable alternative would be to engineer a biosynthetic pathway for omega-3 LCPUFA-production into a land-based host, such as a commercial oilseed crop. Since most cold water marine fish oils have very low levels of omega-6 fatty acids (2–5%) and a combined omega-3 LCPUFA content of 10–25% (EPA + DHA), this composition provides a suitable commercial target for omega-3 LCPUFA in plants.

Engineering omega-3 LCPUFA into Plants: Polyketide Biosynthetic Pathways

Although many cold-water fish are capable of synthesizing LCPUFA from LNA and ALA, the typically high content of EPA and DHA found in their body oils can only be attained through their dietary intake of LCPUFA [27]. For large marine carnivores, such as tuna and salmon, substantial amounts of LCPUFA are obtained by eating smaller fish, such as Menhaden. For the small fish the main sources of LCPUFA are marine microorganisms such as diatoms, golden-brown algae, green algae, blue-green algae, microbial fungi and dinoflagellates, all of which are rich in LCPUFA synthesized *de novo* by one of two classes of biochemical pathway. These two classes are the anaerobic polyketide synthase pathways [28] and the aerobic fatty acid desaturation/elongation pathways [29].

Polyketides are a very broad group of secondary metabolites that are usually defined by their method of synthesis; that is, the iterative addition of carbon to a growing acyl-ACP chain catalyzed by a single enzyme complex known as a polyketide synthase (PKS). These PKS complexes catalyze reactions analogous to those of fatty acid metabolism [30]. But whereas aerobic fatty acid synthesis is the result of over 30 distinct enzyme activities, a PKS synthase consists of a single, multidomain enzyme with subunits encoded by only three or four open reading frames in the genome of the polyketide-producing organism [30]. While most PKS products, such as aflatoxins and antibiotics, are highly derivatized and cyclized acyl chains some marine organisms use PKS-type complexes to synthesize EPA or DHA. In general, the species of LCPUFA produced by these organisms is specific to the particular polyketide synthase they contain [28]. In some cases, a single organism may contain both PKS and fatty acid synthase pathways for EPA or DHA synthesis. For example, a complete PKS type DHA-synthase has been cloned and characterized from a number of Thraustochytid species, as have various fatty acid synthase-type enzymes involved in EPA and DHA synthesis [31, 32].

Genes encoding the three subunits of a *Schizochytrium* PKS that catalyzes the synthesis of DHA from malonyl-CoA have been expressed in yeast. When co-expressed with a phosphopantetheinyl transferase (PPT) from *Nostoc*, essential for activating the ACP domains of the DHA-synthase PKS, the yeast are able to produce small amounts (2.5%) of DHA [33]. Presumably, the ultimate intent is to transfer the *Schizochytrium* PKS and *Nostoc* PPT genes into an oilseed plant with the goal of producing DHA in the seed oil, although there are no published reports to date of the success or otherwise of this approach.

Engineering omega-3 LCPUFA into Plants: Fatty Acid Biosynthetic Pathways

In contrast to PKS synthases, the pathways of aerobic ARA, EPA and DHA synthesis use a series of individual desaturase and elongase activities to catalyze the conversion of LNA and ALA to LCPUFA [34]. For ARA and EPA synthesis this requires the addition of two carbons and two double-bonds to LNA and ALA respectively. ARA can be converted to EPA by the action of a third (omega-3) desaturase [35].

Two converging ARA/EPA pathways have been identified in LCPUFA-producing organisms (Fig. 1); in both pathway types LNA and ALA are the metabolic precursors. In the first pathway type, LNA and ALA are first desaturated to gamma-linolenic acid (GLA, 18:3 [6, 9, 12]) and stearidonic acid (STA, 18:4 [6, 9, 12, 15]), respectively, by a delta-6 fatty acid desaturase. These fatty acids are then elongated to 20-carbons by a microsomal fatty acid elongation complex [28]. This elongation is initiated by a delta-6 specific beta-ketoacyl-CoA synthase enzyme (delta-6 elongase). The 20-carbon ketoacyl-CoA is then reduced, dehydrated and reduced again by the elongation complex to yield dihomo-gamma-linolenic acid (DGLA, 20:3 [8, 11, 14]) or eicosatetraenoic acid [ETA, 20:4 [8, 11, 14, 17]). These fatty acids are then desaturated to ARA and EPA respectively by a delta-5 desaturase.

In the second pathway type, LNA and ALA are first elongated by a delta-9-specific elongase to eicosadienoic acid (EDA, 20:2 [11, 14]) and eicosatrienoic acid (ERA, 20:3 [11, 14, 17]), followed by delta-8 desaturation to DGLA and ETA, respectively. As in the first pathway, these fatty acids are then desaturated to ARA and EPA, respectively by a delta-5 desaturase.

Independent of the aerobic pathway utilized, some organisms have the added capability of efficiently converting omega-6 fatty acids to omega-3 fatty acids by the action of an omega-3 fatty acid desaturase [35–39]. This desaturation can occur on either 18-carbon or 20-carbon fatty acids.

In most organisms, conversion of EPA to DHA occurs by delta-5/C20 elongation of EPA to docosapentaenoic acid (DPA, 20:5 [7, 10, 13, 16, 19]) followed by delta-4

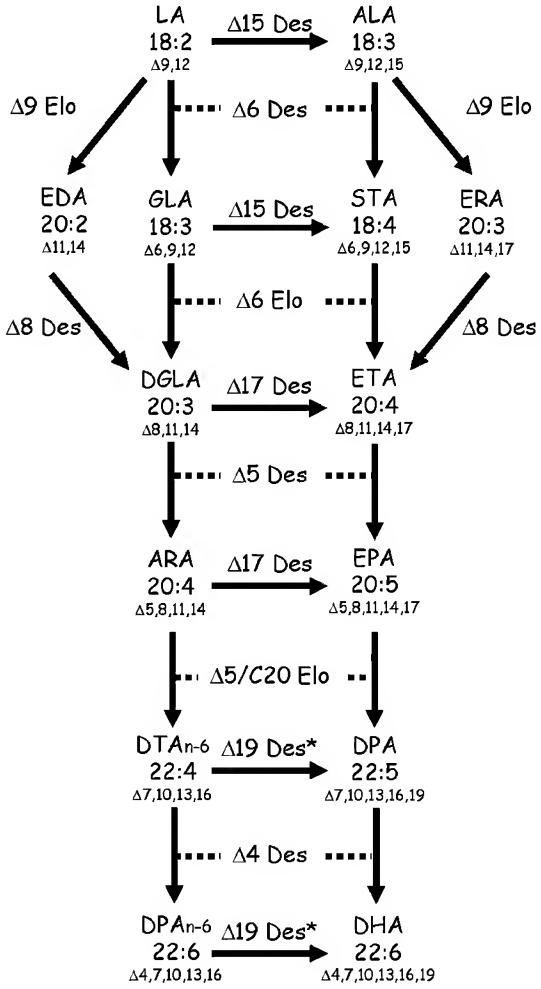

Fig. 1 Aerobic LCPUFA biosynthetic pathways in marine microbes. The delta-6 pathway is shown with a delta-6 desaturase (*Δ6 Des*) and delta-6 elongase (*Δ6 Elo*) and the delta-9 pathway is shown with a delta-9 elongase (*Δ9 Elo*) and a delta-8 desaturase (*Δ8 Des*) Both pathways utilize a delta-5 desaturase (*Δ5 Des*). All pathway enzymes utilize both omega-3 and omega-6 fatty acid substrates and this is indicated with a *dotted line*. Conversion of 18 carbon, 20 carbon or 22 carbon omega-6 fatty acids to omega-3 fatty acids is catalyzed by the delta-15 desaturase (*Δ15 Des*), delta-17 desaturase (*Δ17 Des*) and possibly by a hypothetical delta-19 desaturase (*Δ19 Des**), respectively. Elongation of ARA or EPA is catalyzed the Δ5/C20 elongase (*Δ5/C20 Elo*) and further delta-4 desaturation by the delta-4 desaturase *(Δ4 Des)*

desaturation to DHA [29]. In humans and some other mammals, DHA synthesis is considerably more complex, involving two further elongations, a desaturation and beta-oxidation in a separate cellular compartment [34].

Among the first published descriptions of expression of a fatty acid-type LCPUFA pathway in plants was a report describing the constitutive expression of a delta-9 elongase pathway using genes from the microalgae *Isochrysis galbana* and *Euglena gracilis* and the microbial fungus *Mortierella alpina* in the model plant *Arabidopsis* [40]. Individual pathway genes (delta-9 elongase, delta-8 desaturase, delta-5 desaturase) were each linked to a Ca35S promoter and resulting EPA contents as high as 3.0% and ARA contents up to 6.6% were produced in *Arabidopsis* leaves. The ratio of omega-3 to omega-6 ratio fatty acids (2.2:1) was slightly lower in the transgenic plants than that for wild-type *Arabidopsis* leaves (3.5:1), but the new ratio was still in the range commonly found in fish oils [22]. A number of pathway intermediates and pathway by-products not commonly found in fish oils were also observed. These results were a significant proof-of-concept for expressing LCPUFA pathways in plants; however, *Arabidopsis* leaves could not be an economic production platform for these lipids.

In another report, published around the same time as the *Arabidopsis* study, Abbadi et al. [41] demonstrated very minor accumulation (less than 2%) of ARA and EPA in tobacco and flax seeds expressing a microbial delta-6 type pathway with genes from the diatom *Phaeodactylum tricornutum* and the fungus *Physcomitrella patens*. As in the *Arabidopsis* study there was substantial accumulation of pathway intermediates, mostly GLA and STA.

Thus, 18-carbon fatty acid elongation appeared to be limiting in the delta-6 pathway experiments (elongation was estimated to be around 10% in both tobacco and flax) but not in the delta-9 pathway experiments (where elongation in *Arabidopsis* was estimated to be about 36%). Poor elongation of delta-6 fatty acids was attributed to the low pool of the delta-6 acyl-CoA (GLA-CoA, STA-CoA) which are the main substrates for the delta-6 elongase. The low delta-6 acyl-CoA pools were probably the result of poor acyl-exchange of these lipids from the phospholipid substrates of the delta-6 desaturase to the acyl-CoA substrates for elongation. The pools of LNA-CoA and ALA-CoA for delta-9 elongation were presumably not limiting in the *Arabidopsis* study since these acyl species are the major fatty acids found in *Arabidopsis* leaf lipids. In a later study [42] the abundance of EPA in *Brassicca juncea* expressing a delta-6 pathway was increased to as high as 15% partly by including a gene encoding a lysophosphatidyl acyltransferase from *Thraustochytrium* sp. This acyltransferase presumably increased the exchange of delta-6 acyl groups for acyl-phospholipids to acyl-CoAs for elongation.

However, a third report from 2004, describing commercially-significant amounts of LCPUFA in plant seeds reported good delta-6 elongation and a high abundance of EPA without the use of additional acyltransferases [43]. In this study, a delta-6 desaturase-type pathway from *M. alpina* was expressed in the agronomically-important oilseed crop soybean, under control of strong, seed-specific promoters. In addition to the delta-6 pathway-genes (delta-6 desaturase, delta-6 elongase, delta-5 desaturase), the omega-6 ratio was increased from 0.2:1 (the normal soybean ratio) to 1.5:1 (a ratio close to that of many fish oils)

by concomitant expression of an *Arabidopsis* Fad 3 gene [44] and a *Saprolegnia diclina* delta-17 desaturase [39, 45].

The soybean study was the result of extensive characterization of multiple seed specific promoters and LCPUFA biosynthetic genes from different microbial sources, as well as optimization of promoter-gene cassette combinations and orientations in soy. Using this approach soybean seeds with an EPA content as high as 19.5% were produced with virtually no ARA. The low ARA content was attributed to the use of the *S. diclina* delta-17 desaturase. Additionally, the DHA precursor, DPA was found in high EPA lines at an abundance of about 4%, a result of the additional activity of the *M. alpina* delta-6 elongase towards the delta-5 fatty acid EPA. This same elongase had virtually no delta-5 EPA-elongating activity when expressed in yeast [46].

As in the other plant studies, pathway intermediates and by-products were present (35% in total). Unlike the flax and tobacco studies however, fatty acid elongation was not limiting in soybean. The total 20-carbon fatty acid content was as high as 40.2% representing 56% elongation of 18-carbon substrates.

In a more recent study, in which a similar set of LCPUFA biosynthetic genes for the same source were expressed (but without the omega-3 desaturases since the intent was to produce ARA), a very low abundance of total LCPUFA was observed (2.1%) in soy embryos [47]. The authors conclude that, as demonstrated in the soybean work of Kinney et al. [43], the careful combination of specific genes with individual promoters, gene cassette design and the screening of numerous events is crucial to obtaining the correct balance and a significant abundance of the desired LCPUFA in plants. Similar conclusions around the need for unique seed-specific promoters and pathway flux optimization were reached by Robert et al. [48] who expressed genes from a species of zebrafish (*Danio rerio*) and *Caenorhabditis elegans* in *Arabidopsis* seeds and observed a total LCPUFA content (ARA + EPA + DHA) of only 4.2%.

The flux to omega-3 fatty acids has since been further improved in soybean embryos [49] by the use of a novel, bifunctional delta-12/delta-15 desaturase from *Fusarium moniliforme* [39] in place of the *Arabidopsis* omega-3 desaturase. In the best events, the overall omega-3 fatty acid content was as high as 57% of total fatty acids. The *Fusarium* delta-15 desaturase was shown to be very active in soybean and when expressed alone, lead to ALA contents as high as 72%. It also had broad substrate specificity for numerous omega-6 fatty acids including LNA > GLA > DGLA > ARA [38], which further increases its usefulness in an LCPUFA pathway. Further LCPUFA pathway flux optimization and reduction in non-target fatty acids has since been achieved by further selection of the types of desaturases and elongases used, codon optimization of microbial genes for plants and engineering optimal enzyme specificities (Damude and Kinney, unpublished data).

Replacing Fish Oils with Transgenic Plant Oils

In the soybean study above, Kinney et al. [43] reported a relative abundance of DHA in soybean somatic embryo oil of up to 3.3%, which was the first demonstration of DHA in an oilseed plant. Soy somatic embryos are equivalent to the zygotic embryos of seeds [50]. This was achieved by the addition of a delta-5 elongase from *Pavlova* sp. [45] and a delta-4 desaturase from *Schizochytrium aggregatum* [51] in addition to the EPA biosynthetic pathway genes described above. The delta-4 desaturase used was highly active in plants with, in some cases, close to 100% conversion of DPA to DHA. In two other studies [42, 48], DHA was produced in a relative abundance of 0.5–1.5%. These are important milestones since all fish oils contain a mixture of EPA and DHA and thus the realization of a plant-based fish oil substitute appears to be on the horizon. Nevertheless, key challenges remain in obtaining a plant-based oil that is substantially similar to other commercially available fish oils. The first target will be to produce an EPA plus DHA plant oil with DHA comprising at least 10% of the total fatty acids, close to that of some marine oils. Although the highest abundance of DHA reported so far is less than 4%, current technology will allow for this abundance to be increased around threefold while maintaining an EPA abundance in the 10–15% range and this will provide an effective marine oils substitute. It will also be important to keep omega-6 fatty acids, pathway intermediates and pathway by-products to a minimum. It is now understood that by achieving the correct balance of relative gene expression and optimum metabolic flux through the engineered pathway it is possible to meet these criteria in commercial oilseed plants. Of course, once a seed oil having the desired target fatty acid composition has been achieved, there remains the key regulatory and agronomic challenges that face all new transgenic crop plants. However, the promising use of plant seed oils modified by biotechnology to produce n-3 long chain fatty acids will provide a readily available source of these important fatty acids in the future, overcoming the problems associated with obtaining n-3 LCPUFA from declining ocean fish supplies.

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Minireview

MARINE BIOTECHNOLOGY

Production of Eicosapentaenoic and Docosahexaenoic Acid-Containing Oils in Transgenic Land Plants for Human and Aquaculture Nutrition

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Abstract

A large body of evidence suggests that there is a significant underconsumption of omega-3, long-chain, polyunsaturated fatty acids (LC-PUFAs) and that this is the cause of multiple chronic diseases and developmental aberrations. The scope for increasing omega-3 LC-PUFA consumption from seafood is limited because global wild fisheries are unable to increase their harvests, and aquaculture fisheries currently rely on wild fisheries as a source of LC-PUFAs. Agricultural production of oils is highly efficient and has the potential to be sustainable. The transfer of genes from marine microalgae and other microorganisms into oilseed crops has shown that the production of terrestrial omega-3 LC-PUFA oils is indeed possible. The specifications of these oils or whole seeds for use in human and Atlantic salmon (*Salmo salar*) aquaculture nutrition are discussed.

Keywords: docosahexaenoic acid — eicosapentaenoic acid — nutrition — transgenic

Medical evidence accumulated over the last two decades strongly demonstrates that too low a consumption of omega-3 long-chain polyunsaturated fatty acids (LC-PUFAs) increases the incidence of cardiovascular disease (Hu and Willett, 2002; Lee and Lip, 2003; Shahidi and Miraliakbari, 2004), cancer (Rose and Connolly, 1999; Wen et al., 2003; Reddy, 2004; Roynette et al., 2004), stroke (Hankey and Jamrozik, 1996), diabetes (Coste et al., 2003; Seo et al., 2005), inflammatory disease (Simopoulos, 2002; Nagel et al., 2003), neuropsychiatric disorders (Reddy and Yao, 2003), and many other conditions prevalent in countries with a Western-style diet (Simopoulos, 2004).

Currently the best source of omega-3 LC-PUFAs is seafood. In developed countries such as Australia, the United States, and the United Kingdom, seafood consumption is very low. In Australia, for example, the median intake of the two most beneficial omega-3 LC-PUFAs, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), is 30 mg/day (Australian Bureau of Statistics, 1995). Although a recommended daily intake (RDI) has not yet been established for EPA and DHA, the guidelines range from 450 mg/day recommended by the UK Food Standards Agency, to 500 mg/day recommended by the International Society for the Study of Fatty Acids and Lipids (ISSFAL), to 650 mg/day of combined EPA and DHA recommended by expert groups from Australia, who indicate that DHA should represent a minimum of 220 mg (Simopoulos et al., 1999; Nichols, 2004).

Besides the cultural issues associated with increasing seafood consumption such that these guidelines are met, there is a serious pragmatic problem. Global wild fisheries have not increased their catch rates over the last decades despite increasing effort (Myers and Worm, 2003). There is ample evidence that most wild fisheries have either reached a plateau or are on the decline (Caddy and Garibaldi, 2000). Globally, aquaculture is growing rapidly; in 2000 its yield was 40% that of wild fisheries but it is expected to surpass the yield of wild fisheries by 2020–2025 (Tacon, 2003). Carnivorous aquacultured species, such as Atlantic salmon (*Salmo salar*), have traditionally relied on omega-3 LC-PUFAs supplied in their diets for development and growth. Currently, these omega-3 LC-PUFAs are supplied as fish oil or are present as residue

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in fishmeal (New and Wijkstrøm, 2002). Both fish oil and fishmeal are extracted from wild-catch fisheries.

Given the decline in fisheries, the increase in aquaculture, the requirement for omega-3 LC-PUFAs, the growing awareness of this requirement, and the growing human population, an alternative source of omega-3 LC-PUFAs must be found. Although crops such as canola, soy, and linseed do not synthesize omega-3 LC-PUFAs, agricultural production of oil from these crops is extremely efficient and has the potential to be sustainable if best agricultural practices are followed. For this reason, several research groups are attempting to engineer omega-3 LC-PUFA synthesis in oilseed crops.

LC-PUFA Biosynthesis Genes

In the marine food web, microalgae and other organisms such as thraustochytrids, which fish consume directly or indirectly, are the primary source of omega-3 LC-PUFAs.

To this end, several sets of genes encoding enzymes in alternative biosynthetic pathways have been isolated from a number of marine microalgae, thraustochytrids, and terrestrial fungi and transferred to several different plants. One set isolated from the thraustochytrid *Schizochytrium* sp. by Metz and colleagues and encoding a modified polyketide synthase (Metz et al., 2001) is not covered here. The remaining LC-PUFA biosynthetic genes isolated are front-end fatty acid desaturases, which introduce double bonds at positions counted from the carboxyl or front end of the molecule, and fatty acid elongases, which add two carbon units to existing fatty acids. Two excellent reviews comparing and contrasting the successes of these engineering attempts have been published during the past year (Domergue et al., 2005; Singh et al., 2005). The intention of this minireview is to summarize the approaches and results before suggesting a way forward, based on a simple analysis of human and Atlantic salmon requirements for LC-PUFAs, with the design of LC-PUFA-containing seeds and oils.

That the production of LC-PUFA in plants is at all possible was first demonstrated by the landmark study of Qi et al. (2004). The authors constitutively expressed an elongase gene from the microalga *Isochrysis galbana* and two desaturase genes—one from the microalga *Euglena gracilis* and one from the fungus *Mortierella alpina*—in the model plant *Arabidopsis thaliana*. This resulted in the production of low but significant levels of EPA and AA in the leaf tissue. Besides showing that the coupling of LC-PUFA biosynthesis with the plant's endogenous fatty acid biosynthesis was indeed possible, this study also demonstrated for the first time that there exists an "alternate" order in which LC-PUFA production by desaturation and elongation can occur. Figure 1 shows that the standard order of desaturation and elongation for 18:3n-3 to produce EPA proceeds via a Δ6 desaturation followed by a Δ6 elongation and then a Δ5 desaturation. Qi et al. (2004) demonstrated, by their discovery of a Δ9 elongase from *Isochrysis galbana* and Δ8 desaturase

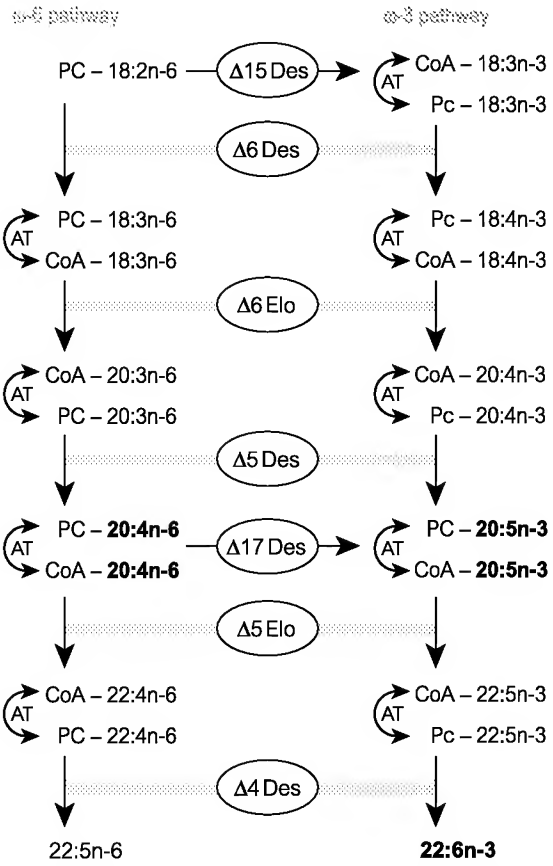

Fig. 1. ω-3 and ω-6 LC-PUFA biosynthetic pathways. LC-PUFA biosynthesis starts with 18:2n-6 (LA) which is either desaturated at the Δ6 position and progresses down the ω-pathway or is desaturated at the Δ15 position to produce 18:3n-3 (ALA), which progresses down the ω-3 pathway. Subsequent desaturation (Des) and elongation (Elo) in each pathway is carried out by the shared enzymes (gray bars) except for the Δ17 desaturase, which converts 20:4n-6 into 20:5n-3. Fatty acids are linked either to phosphatidylcholine (PC) or coenzyme A (CoA). All desaturations occur to fatty acids coupled to PC while elongations occur to fatty acids coupled to CoA. The "shuttling" of fatty acids from PC to CoA pools is achieved by acyltransferases (AT). The key fatty acid products of the pathways, **20:4n-6** (AA), **20:5n-3** (EPA), and **22:6n-3** (DHA), are highlighted in bold font.

8 desaturase from *Euglena gracilis*, that it is possible to first Δ9 elongate 18:3n-3 and then Δ8 desaturate it on the way to producing EPA.

While this study itself is of great importance, it also encouraged researchers to pursue the ultimate goal—production of EPA and DHA specifically in the seed oils of plants.

Many higher plants synthesize 18:2n-6 (linoleic acid [LA]) and 18:3n-3 (α-linolenic acid [ALA]) in their seed oils but do not produce fatty acids further elongated or desaturated. To make 20:5n-3 (eicosapentaenoic acid [EPA]) or 22:6n-3 (docosahexaenoic acid [DHA]) in seed oils, addition of up to two elongation and three desaturation activities is required (Figure 1). As can be seen in Figure 1, these enzymes can also act on omega-6 precursors and produce the long-chain omega-6 fatty acid 20:4n-6 (arachidonic acid [AA]).

Abbadi et al. (2004) transferred a Δ6 desaturase and a Δ5 desaturase from the diatom *Phaeodactylum tricornutum* as well as a Δ6 elongase from the fungus *Physcomitrella patens* into flax (*Linum usitatissimum*) (see Table 1) in order to produce EPA (see Figure 1). They were able to produce low but significant levels of AA (1.5%) and EPA (1.0%) (Table 1) in the seed, presumably by the action of the introduced genes on the precursor fatty acids LA and ALA, respectively. The authors speculated that the reason for the low levels observed was probably that the algal desaturases use phosphatidylcholine (PC)-linked fatty acids as substrates while elongation occurs on fatty acids esterified to coenzyme A (CoA). In flax, the seed triacylglycerols (TAGs) are rich in ALA and, to a lesser extent, LA. As such, the authors suggest that LA and ALA are shunted efficiently into the acyl-CoA pool, by the action of a presumptive acyl transferase (AT) (Figure 1), prior to incorporation into TAG. This would make only low levels available in the PC pool for the first desaturation step by the introduced Δ6 desaturase and subsequently reduce the total yield of EPA and AA. Furthermore, the authors speculate that ATs that can efficiently transfer 18:3n-6 and 18:4n-3 from the PC pool to the CoA pool for a subsequent elongation and ATs that can transfer 20:3n-6 and 20:4n-3 from the CoA pool to the PC pool for a subsequent Δ5 desaturation do not exist in flax and the endogenous ATs may have a low affinity for these substrates.

In an attempt to circumvent the problems with acyl shuttling between PC and CoA pools observed by Abbadi et al. (2004), Robert et al. (2005) utilized fatty acid desaturases that act on acyl moieties coupled to CoA. In addition, their aim was to go one biochemical step further and produce DHA in seeds. To this end, they transferred four genes into the thale cress *Arabidopsis thaliana*, each under the control of the seed specific promoter from the *Napin* gene. These were a dual activity Δ5/Δ6 desaturase from the zebrafish *Danio rerio*, an elongase with Δ6 activity from the nematode *Caenorhabditis elegans*, and a Δ5 elongase and a Δ4 desaturase from the prymnesiophyte *Pavlova salina*. These authors observed low levels of AA (1.2%), EPA (2.5%), and DHA (0.5%) (Table 1). They speculated that the multiple use of the same

Table 1. Summary of Genes, Host Plants, and Reported LC-PUFA Proportions in Seeds of Transgenic Plants

	Abbadi et al. (2004)	*Kinney et al. (2004)*	*Robert et al. (2005)*	*Wu et al. (2004)*
Genes	*Pt Δ6Des* *Pt Δ5Des* *Pp Δ6Elo*	*Sd Δ6Des* *Ma Δ6Des* *Ma Δ5Des* *Sa Δ4Des* *Sd Δ17Des* *Ma Δ6Elo* *Pav Δ5Elo*	*Dr Δ5/6Des* *Ps Δ4Des* *Ce Elo* *Ps Δ5Elo*	*Pi Δ6Des* *Th Δ5Des* *Th Δ4Des* *Co Δ12Des* *Pin Δ17Des* *Om Elo* *Pp Δ6Elo* *Th Δ6Elo* *Th LPAAT*
Host plant	*L. usitatissimum*	*G. max*	*A. thaliana*	*B. juncea*
AA	1.5		1.2	25
EPA	1.0	19.6	2.5	15
DHA	Na	2–3.3	0.5	1.5

Numbers shown are highest levels reported in any line expressed as percentage of total fatty acids in seeds.
Ce, *Caenorhabditis elegans*; Co, *Calendula officinalis*; Dr, *Danio rerio*; Ma, *Mortierella alpina*; Om, *Onchorhynchus mykiss*; Pav, *Pavlova* sp.; Pi, *Pythium irregulare*; Pin, *Phythophtora infestans*; Pp, *Physcomitrella patens*; Ps, *Pavlova salina*; Pt, *Phaeodactylum tricornutum*; Sa, *Schizochytrium aggregatum*; Sd, *Saprolegnia diclina*; Th, *Thraustochytrium* sp. 26185.
AA, arachidonic acid; Des, desaturase; DHA, docosohexaenoic acid; Elo, elongase; EPA, eicosapentaenoic acid; LPAAT, lysophospatidic acid acyl transferase.
na, not applicable.

promoter as well as the possible low level of 18:3n-3 available for Δ6 desaturation contributed to low gene expression levels and LC-PUFA biosynthesis respectively.

Prior to the study of Robert et al. (2005), but publicly unknown due to the lag between patent submission and publication, Kinney et al. (2004) were successful in producing high levels of LC-PUFA (19.6% EPA, 2% to 3.3% DHA; see Table 1) in soy (*Glycine max*) seed and embryos. This group transferred multiple genes, predominantly from fungi, to achieve EPA synthesis in soybean seed and DHA synthesis in regenerated soy embryos (see Table 1). Most interesting was their use of a Δ17 desaturase that was able to shunt the majority of biosynthesis in the omega-6 pathway over to EPA in the omega-3 pathway (see Figure 1). In this way desirable omega-3 production was increased at the expense of less desirable AA production and in spite of the lack of selectivity of the other desaturases and elongases used for omega-3 fatty acids as substrates (see Figure 1). Clearly, soy is also a much better vehicle for LC-PUFA production than flax or *Arabidopsis* for several reasons pertaining to substrate availability (Domergue et al., 2005).

Most recently, Wu et al. (2005) have transferred a large number of genes (see Table 1) into rapeseed (*Brassica juncea*) and produced high levels of AA (25%; Table 1) and EPA (15%) and low levels of DHA (1.5%; Table 1). Using the same method as Kinney et al. (2004), Wu et al. (2005) used a Δ17 desaturase to shunt a large amount of AA into the n-3 pathway at EPA (Figure 1). Interestingly, the ratio of DHA produced relative to EPA was the lowest of all three studies at 0.1, whereas Kinney et al. (2004) reported 0.17 and Robert et al. (2005) showed the highest ratio of 0.2. This suggests that the availability of EPA for further elongation and desaturation into DHA may vary depending on the host plant or that the efficiency of the Δ5 elongases used in the studies was different.

Given the variation in levels of EPA and the relatively low levels of DHA produced in the aforementioned studies, the question that must be asked is what levels are desirable? Is there one ideal fatty acid profile, with respect to amounts and ratio of EPA and DHA, for human or aquaculture nutrition?

Human Nutrition

If a commercially available vegetable-based omega-3 LC-PUFA oil were to be marketed as a dietary supplement, a good starting point would be to compare these plant oils with widely used fish oil supplements. The most common fish oil supplement, extracted from Northern hemisphere herring, menhaden, sardines, and other species, is known as an 18:12 oil. It contains 180 mg (18%) of EPA and 120 mg (12%) of DHA per 1000 mg of oil. At the current recommended intakes of 450 to 650 mg of combined EPA and DHA, 1.5 to 2.2 grams of oil would need to be consumed daily.

The EPA content of the oils produced by Kinney et al. (2004) and Wu et al. (2005) compares favorably with the 18:12 fish oil at 19.6% and 15%, respectively (Table 1). DHA, however, is at a lower level in both studies. Wu et al. (2005) report a maximal 1.5% and Kinney et al. (2004) report a maximal 3.3%. This is between 8- and 3.6-fold less, respectively, than that found in fish oil. It is now known that DHA and EPA have different roles in human nutrition (Horrocks and Yeo, 1999; Madsen et al., 1999; Mori et al., 1999, 2000). Given this and the slow enzymatic conversion of EPA to DHA in humans (Hodge et al., 1993), it might be important to maintain the 18:12 ratio of EPA to DHA in a vegetable oil or even increase the proportion of DHA. In either case, the ratio of EPA to DHA reported by Wu et al. (2005) and Kinney et al. (2004) should be reduced. It must be noted, however, that while the recommended intakes prescribe a combined amount of EPA and DHA, only the Australian panel recommends an individual DHA intake of 220 mg/day (Simopoulos et al., 1999). Relative to the recommended combined intake of 650 mg/day, this translates to an oil with an EPA to DHA ratio of 430:220 or close to 2:1—higher than that found in 18:12 fish oils. Further nutritional studies will be required to determine if ratios of EPA to DHA higher than the 18:12 found in fish oils are indeed adequate and beneficial for human health.

An alternative way of increasing omega-3 consumption in the population is to introduce these nutrients into commonly eaten foods. This is currently being done by the addition of microencapsulated fish oils to various food products such as bread, juice, and dairy products (e.g., Yep et al., 2002). In the future, it should be possible in food manufacture to simply utilize commodity crops that have been transformed with the genes that make omega-3 LC-PUFAs. If this were to occur it would be important to determine the levels of omega-3 LC-PUFAs in oils and grains that would be desirable such that a "normal" diet would provide an adequate amount of these nutrients. Consider two examples of high-oil food products that might incorporate omega-3 fatty acids—mayonnaise and margarine. A standard serving size of regular commercial mayonnaise is 1 tablespoon or 14 grams.

This contains 12 grams of fat, derived mostly from vegetable oils. To obtain a 650-mg dose of combined EPA and DHA, the oil used to produce the mayonnaise should contain a combined proportion of EPA and DHA of approximately 5.5%. A single serving of margarine is 5 grams, of which approximately 3.75 grams is fat derived from vegetable oils. To obtain 650 mg of combined EPA and DHA the oil used to make the margarine should contain approximately 17.5% EPA and DHA combined. Clearly, these values are at the high end. 650 mg is the maximum currently recommended for omega-3 LC-PUFA consumption and, more importantly, consuming multiple food products over the course of the day enriched with these nutrients would mean that even lower levels of omega-3 LC-PUFAs in these products would be adequate. In this scenario, perhaps oils with omega-3 LC-PUFA percentages less than 5% would be desirable in the formulation of high-oil foods.

Of course, food manufacturers might prefer to have access to a high concentration of omega-3 LC-PUFA oils that they can add to formulate foods with desired omega-3 content. Technical issues such as oxidative stability or germination problems may disallow this.

Therefore, going forward, low omega-3 LC-PUFA-containing oils might be the least expensive, safest, and most practical way for use in food products.

Aquaculture Nutrition

Currently, the major source of omega-3 LC-PUFAs in human diets is seafood. To maintain the nutritional status of seafood, aquaculture feeds based on plant-derived oils must maintain high levels of omega-3 LC-PUFAs in animals at harvest. Current formulated feeds for Atlantic salmon, for example, do this, but in the quest to replace fish oils with transgenic plant oils, seafood quality must be maintained.

Previous studies have shown that Atlantic salmon has the same growth rates and health status when reared on diets formulated with fish oil or sunflower oil, which contains no EPA or DHA. However, the fatty acid composition of the flesh begins to resemble that of the feed oil such that sunflower oil-fed fish contain higher levels of undesirable linoleic acid (LA, 18:2n-6) and lower levels of EPA and DHA (Bransden et al., 2003). That the salmon fed sunflower oil still contain some EPA and DHA suggests that they may be able to synthesize these compounds *de novo*. This is supported by fact that salmon carry all the genes necessary for biosynthesis of EPA and DHA (Hastings et al., 2004) and data that show that when salmon are fed canola oil diets they upregulate the expression of Δ5 and Δ6 desaturases and synthesize more EPA and DHA than salmon fed on fish oil diets (Zheng et al., 2004, 2005). Studies from Norway on fish fed canola oil suggest that these fish, although containing EPA and DHA, have a lower human nutritional benefit with respect to cardiovascular health (Seierstad et al., 2005). Therefore, while it is important to maintain levels of EPA and DHA in salmon it might be possible to reduce the amount of EPA and DHA in the feed oils and rely on the salmon's own biosynthetic capability. To this end several groups have tried to determine the optimal blend of vegetable oils and fish oils for formulated salmon feeds. Bell et al. (2004) designed feeds in which they substituted linseed oil—rich in ALA—for fish oil. They found that 50% substitution, the lowest they tested, resulted in DHA and EPA levels in the flesh of salmon 65% and 58%, respectively, of those observed when fish are fed 100% fish oil. They found that a subsequent finishing diet of 100% fish oil for 16 weeks returned flesh EPA and DHA to control levels. Bransden et al. (2003) found that they could replace 40% of the fish oil with sunflower oil and make no significant difference to the levels of DHA in salmon flesh. If new omega-3, LC-PUFA-containing vegetable oils can be made available, their EPA and DHA content need not match fish oils but resemble that found in these vegetable/fish oil blends. Using the 40% substitution level (Bransden et al., 2003) would mean that a transgenic vegetable oil with 60% of the DHA and EPA—7.2% and 10.8% respectively—of fish oil could be adequate to maintain the high levels of these fatty acids that make salmon nutritionally desirable. It is important to note that such an oil should be low in undesirable omega-6 PUFA as these would also become incorporated into the salmon flesh and increase the omega-6:omega-3, reducing health benefits regardless of the absolute levels of EPA and DHA (Simopoulos, 2004).

Conclusion

Remarkable progress in engineering omega-3 LC-PUFA production in land plants has been made by several groups in the last few years. Notably, the levels of total PUFA, EPA, and DHA production have been variable and reflect the choice of transgenes, promoters, and host plants. Although the ultimate aim of these researchers was to produce a fish oil substitute, none of the currently produced oils exactly matches the fatty acid composition of fish

oil. While the rapeseed oil (Wu et al., 2005) and soy oil (Kinney et al., 2004) have levels of EPA resembling fish oil, their DHA levels are much lower. The importance of an 18:12 EPA to DHA ratio, as found in commercial fish oils, may not be critically important but further studies are required to resolve this.

Oils that contain low levels of EPA and DHA (less than 5%) could be useful in food production because of probable stability and processing advantages and contribute to the cumulative consumption of recommended daily amounts omega-3 LC-PUFA from multiple enriched food products. These low-level oils may also offer the best chance at reproducing an 18:12 EPA to DHA ratio if deemed necessary. As far as replacing fish oils in salmon feeds, transgenic oils with proportions of PUFA as low as 10.8% EPA and 7.6% DHA may be adequate for maintaining fish nutritional quality without the need for finishing diets (Bell et al., 2003).

This strongly suggests that transgenic omega-3 vegetable oils need not match fish oils in order to find utility.

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REVIEW

Progress towards the production of very long-chain polyunsaturated fatty acid in transgenic plants: plant metabolic engineering comes of age

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Very long-chain polyunsaturated fatty acids are vital components of the human diet, playing important multiple roles in optimal health and development. Current dietary sources, in the form of fish oils, are in decline, and an alternative sustainable source is required. Recent attempts to engineer transgenic plants with the capacity to synthesize these fatty acids have clearly demonstrated the possibility of such an approach. This represents a major breakthrough in the quest for alternative sources of fish oils, as well as fulfilling the promise of transgenic plants as green factories.

Introduction

As discussed in this Volume, transgenic plants have attracted considerable interest as potential 'green factories' for the synthesis of useful compounds in a sustainable and cheap manner (Thelen and Ohlrogge 2002). Much of this interest is based around the fact that plants are effectively carbon-neutral (thus not contributing to CO_2 emissions), and the requirements for plant growth are minimal and cheap (e.g. sunlight, water). Over the last two decades, the advent of plant genetic engineering has facilitated the mobilization of 'traits' (i.e. genes) from species not normally amenable to conventional breeding programs. This includes not only the transfer of traits between distinct plant families but also the introduction of genes from non-plant species. A well-known successful example of this latter approach is the *Bacillus thuringiensis* insecticidal crystal protein gene (*Bt cryIAc*) which has been shown to confer insect resistance to transgenic plants expressing this bacterial gene (Estruch et al. 1997).

Transgenic crop plants are often categorized as containing 'input' or 'output' traits, terminology which reflects whether or not the transgenic trait confers a benefit to the producer or to the end user (Tucker 2003). In that respect, the *Bt cryIAc* trait would be considered an input trait, because it primarily benefits the farmer by lowering losses to herbivory and dependence on insecticides (although obviously reduced use of pesticides is also of considerable benefit to the general public). It is probably fair to say that most of the first generation of transgenic crops produced over a decade ago was engineered to contain input traits (such as pesticidal properties, herbicide tolerance), which has led to a perception of transgenic plants as solely delivering benefits to the farmer, rather than to the consumer. However, the last few years have seen the development

Abbreviations – ALA, α-linolenic acid; ARA, arachidonic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; GLA, γ-linolenic acid; LA, linoleic acid; LPCAT, acyl CoA lyso-phosphatidylcholine acyltransferases; SDA, stearidonic acid; TAG, triacylglycerol; VLC-PUFAs, very long-chain polyunsaturated fatty acids.

This article is dedicated to Professor Ernst Heinz, who has pioneered work on the production of VLC-PUFAs in transgenic plants.

of second generation transgenic crops which are predominantly engineered to contain output traits. The majority of these are alterations (or more correctly, enhancements) to the nutritional content of plant foodstuffs (Tucker 2003). One of the reasons why these second-generation output traits have taken considerably longer to develop than the input traits is also the increased complexity of the system under manipulation. For example, insect-resistant rice only requires 1 gene (*Bt cryIA*), whereas rice with altered Vitamin A content (Golden Rice) requires several transgenes to be expressed in a co-ordinated manner (Grusak 2005). Thus, it is only now that the promise of second generation transgenic crops is starting to emerge.

One nutritional enhancement output trait that has been the focus of considerable recent interest is the synthesis and accumulation of *n-3* very long-chain polyunsaturated fatty acids (*n-3* VLC-PUFAs) (Drexler et al. 2003). These fatty acids are found predominantly in fish oils and have historically been associated with health-beneficial properties to humans. Beyond such anecdotal evidence, a large body of clinical, genetic and controlled dietary intervention studies over the last 20 years have confirmed the importance of *n-3* VLC-PUFAs to a range of human conditions and pathologies (Burr et al. 1989). For example, the VLC-PUFAs play an important role in neonatal growth and development, in particular brain and eye function (it is for this reason that most replacement formula milks now contain these fatty acids) (Graham et al. 2004). There is also evidence that *n-3* VLC-PUFAs can play a health-protective role in the prevention of cardiovascular disease and associated precursor conditions collectively known as the metabolic syndrome (Nugent 2004). Unfortunately, global fish stocks are in considerable decline, and because this valuable natural resource represents the current predominant source of VLC-PUFAs, an alternative sustainable source of these fatty acids is urgently required. One rapidly emerging contender for this role is transgenic plants that have been engineered with genes encoding the primary biosynthetic pathway for VLC-PUFA biosynthesis (Abbadi et al. 2001). Considerable progress towards the laudable objective of making 'fish oils' in transgenic oilseeds has been made in the last few years, and this review will focus on the latest breakthroughs in this area. Very recent data have confirmed the earlier promise of using transgenic plants as green factories for the synthesis of VLC-PUFAs, serving also as an important validation of second-generation output trait transgenics.

The production of VLC-PUFAs in transgenic plants

It is not the aim of this review to reiterate the enzymes and genes which underpin the biosynthesis of VLC-PUFAs, because this topic has been covered by several recent reviews (Sayanova and Napier 2004, Napier et al. 2003, Sperling et al. 2003). However, for clarity, we will briefly outline the key enzyme reactions. Aerobic VLC-PUFA biosynthesis is catalysed by an alternating sequence of desaturation and elongation reactions (although an unrelated anaerobic pathway exists in some aquatic microbes; Metz et al. 2001). The substrates for VLC-PUFA biosynthesis are linoleic acid (LA; 18:2, *n-6*) and α-linolenic acid (ALA; 18:3, *n-3*). The predominant aerobic PUFA biosynthetic pathway is initiated by the action of the Δ6-desaturase which converts LA and ALA to γ-linolenic acid (GLA; 18:3, *n-6*) and stearidonic acid (SDA; 18:4, *n-3*), respectively. The introduction of the double bond at the Δ6 position occurs via 'front-end' desaturation (i.e. the insertion of a double bond between pre-existing unsaturations and the carboxyl terminus of the fatty acid), and this process is characteristic of aerobic VLC-PUFA biosynthesis. Following desaturation, the Δ6-desaturated fatty acids become substrates for elongation via the microsomal elongase, which extends the acyl chain by two carbons through a condensation reaction with malonyl-CoA. These C_{20} elongation products (20:3, *n-6*; 20:4, *n-3*) are then further desaturated by the Δ5-desaturase to yield arachidonic acid (ARA; 20:4, *n-6*) and eicosapentaenoic acid (EPA; 20:5, *n-3*), respectively. In some unicellular organisms, a so-called 'alternative pathway' exists for the synthesis of these C_{20} VLC-PUFAs, in which the elongation step precedes the first desaturation. In this case, LA and ALA are elongated by a specific C_{18} Δ9-elongase to yield 20:2, *n-6* and 20:3, *n-3* which are then desaturated by a Δ8-desaturase to yield 20:3, *n-6* and 20:4, *n-3*. These two products then undergo Δ5-desaturation as for the conventional Δ6-pathway to yield ARA and EPA. The process of alternating desaturation and elongation also underpins the synthesis of docosahexaenoic acid (DHA; 22:6, *n-3*), although this varies between lower organisms and mammals. In the former case, EPA is elongated to docosapentaenoic acid (DPA; 22:5, *n-3*) by a specific Δ5-elongase, with DPA then converted to DHA by the action of a Δ4-desaturase (Qiu 2003). In mammals, the process is somewhat more complicated and does not use a Δ4-desaturase; EPA undergoes two rounds of elongation, generating first DPA and then tetracosahexaenoic acid (THA; 24:5, *n-3*) with this later C_{24} fatty acid being subject to Δ6-desaturation to yield 24:6, *n-3*. The Δ6-desaturase enzyme that carries out this desaturation is now believed to be the same one as produces GLA and SDA, although the basis for this substrate selectivity is unclear. What is known is that THA undergoes peroxisomal β-oxidation to generate DHA,

although again the mechanism by which this chain shortening is controlled is still unclear. Thus, the biosynthesis of DHA in animals such as humans is considerably more complicated than that observed in unicellular organisms, involving several different compartments (endoplasmic reticulum, peroxisome) (Qiu 2003). In that respect, the ability of mammals to synthesize DHA is perhaps more 'fragile' (or at least more highly regulated), underlying the importance of obtaining this fatty acid in our diet.

The past few years have seen the cloning and functional characterization of all the above primary activities associated with VLC-PUFA biosynthesis (see Sayanova and Napier 2004 for a recent review). This has revealed that all the front-end desaturases associated with PUFA biosynthesis are members of the cytochrome b_5 fusion superfamily, with this electron donor domain at the N-terminal portion of the enzyme (Napier et al. 2003, Sperling et al. 2003). In the case of VLC-PUFA elongation, this is carried out by elongating activities which are members of the ELO/SUR4 superfamily, with these polypeptides assumed to be the condensing enzymes of the microsomal elongase (Leonard et al. 2004). Several key points need to be emphasized regarding VLC-PUFA biosynthetic enzymes. Firstly, the desaturases display differences in their acyl-substrates; in the case of mammalian front-end desaturases, these are assumed to use acyl-CoA substrates (Domergue et al. 2003). In contrast, desaturases from lower eukaryotes use glycerolipid-linked substrates, in which the substrate fatty acid is usually present at the *sn-2* position of phosphatidylcholine (PC) (Domergue et al. 2003). In the case of the elongating activities, the ELO proteins are known to utilize acyl-CoA substrates, although the precise mechanism by which the CoA-substrate is presented to the elongase remains to be elucidated. Finally, while the primary biosynthetic enzymes (desaturases, elongating activities) have all been identified, the contributions of many additional activities remain to be determined. For example, enzymes involved in acyl exchange (acyltransferases, lipases, acyl-CoA synthetases) may play important roles in the efficient synthesis of VLC-PUFAs. With regards to such activities, ongoing efforts are focussed on the identification and functional characterization of the genes encoding such enzymes (see below for discussion).

Proof-of-concept studies

The last year has seen several key demonstrations of the feasibility of the synthesis and accumulation of VLC-PUFAs in transgenic plants, although earlier studies had shown that it was possible to accumulate to high levels C_{18} precursors such as GLA (through the expression of a Δ6-desaturase) (Sayanova et al. 1997). In considering the significance of the observed levels of transgene-derived VLC-PUFAs, it is perhaps appropriate to consider 'ideal' target levels and composition; for example, marine fish oils usually contain 10–20% EPA and DHA combined, whereas the marine microalgae which are primary producers of these VLC-PUFAs can accumulate similar levels for the individual fatty acids (Abbadi et al. 2001, Drexler et al. 2003). Thus, in terms of both biotechnological efficacy and nutritional enhancement, it is desirable to accumulate VLC-PUFAs to the range 10–25%. Such a target range of accumulation seems eminently feasible, not least of all given the earlier studies that reported accumulation of GLA and Δ5-desaturated fatty acids to high levels (Sayanova et al. 1997, Knutzon et al. 1998).

The first report of the successful accumulation of C_{20} VLC-PUFAs in transgenic plants was reported by Qi et al. (2004). In this study, both EPA and the *n-6* VLC-PUFA ARA were shown to be synthesized in transgenic Arabidopsis plants, accumulating to 3.0 and 6.6%, respectively. These C_{20} fatty acids accumulated in the vegetative tissues of the transgenic lines, because the transgenes were expressed under the control of the constitutive CaMV35S promoter. Although the achieved levels of VLC-PUFAs were somewhat lower than the optimal target range, the fact that these fatty acids were synthesized and accumulated with no deleterious effects to the host transgenic plant demonstrated an important 'proof-of-concept'. A number of additional points regarding the data reported by Qi et al. (2004) also need to be highlighted. Firstly, the biosynthetic pathway used to carry out this 'reverse-engineering' (Napier et al. 2004a) of the VLC-PUFA pathway was not the conventional Δ6-desaturase/elongase route but the alternative Δ9-elongase/Δ8-desaturase system. In that respect, the authors speculated that the relative success of their approach was derived from the ability of the (initiating) Δ9-elongase to utilize endogenous LA and ALA substrates present in the acyl-CoA pool. This in turn would provide C_{20} substrates for the subsequent desaturases and be less dependent on the 'switching' of substrates from glycerolipids and acyl-CoA pool (see Napier et al. 2004a, b for discussion of this topic in greater detail). Secondly, the final transgenic lines accumulating ARA and EPA were the result of three sequential genetic transformations, in which the Δ9-elongase, Δ8-desaturase and Δ5-desaturase were introduced as separate transgenes and integration events. Finally, in addition to the accumulation of ARA and EPA, some non-methylene interrupted C_{20} fatty acids were detected, namely sciadonic acid (20:3 Δ5,11,12, *n-6*) and juniperonic acid (20:4 Δ5,11,14,17, *n-3*). While these fatty acids have recently also been considered to

have health-beneficial properties (Nakane et al. 2000), their accumulation was unexpected and likely represents the 'promiscuous' activity of the C_{20} Δ5-desaturase. However, the collective data of Qi et al. (2004) represents a key break-through in the demonstration of VLC-PUFA synthesis in transgenic plants, and serves as the benchmark by which other studies can be compared.

In that respect, the study of Abbadi et al. (2004) provides a very useful accompaniment to that of Qi et al. (2004). Abbadi et al. (2004) expressed the genes for the conventional Δ6-pathway (Δ6-desaturase, Δ6-elongase, Δ5-desaturase) in transgenic linseed under the control of seed-specific promoters and observed very high levels (>25% total fatty acids) of Δ6-desaturated fatty acids (GLA, SDA) but only low levels of the subsequent elongation products, with ARA and EPA accumulating to 0.9 and 0.8% of total seed fatty acids. Thus, it was concluded that there was a 'bottleneck' preventing the elongation step. Additional detailed lipid analysis provided evidence as to the nature of the bottleneck, as profiling of the acyl-CoA pool indicated the almost complete absence of C_{18} Δ6-desaturated fatty acids GLA and SDA, but in vitro biochemical assays confirmed the activity of the heterologous (transgene-derived) Δ6-elongase. Thus, the very inefficient elongation of GLA and SDA was almost certainly due to the absence of these fatty acids in the acyl-CoA pool, which in turn was likely to be due to poor acyl-exchange with phospholipids. This was further confirmed by the observation of specific accumulation of GLA at the *sn-2* position of PC, consistent with earlier studies that demonstrated that many non-mammalian front-end desaturases use glycerolipid-linked substrates (Domergue et al. 2003). In addition, it was observed that C_{18} *n-3* fatty acids (e.g. ALA, SDA) were selectively channelled from PC into triacylglycerol (TAG) in an apparently acyl-CoA independent manner (as witnessed by the absence of these fatty acids in the CoA pool but their accumulation at the *sn-3* position of TAG). This reaction was most likely catalysed by phospholipid DAG acyltransferase (PDAT), and one undesirable consequence of this strong activity in linseed is the further exclusion of the transgene-derived SDA from the acyl-CoA pool, precluding efficient elongation of this fatty acid to a C_{20} PUFA. Thus, although linseed was chosen as a suitable host for the reverse-engineering of *n-3* VLC-PUFA synthesis on the basis of very high levels of the precursor substrate ALA, endogenous channelling activities hindered the accumulation of *n-3* fatty acids such as EPA (Abbadi et al. 2004, Domergue et al. 2005a). Thus, it seems that not only are there bottlenecks that are generic to VLC-PUFA biosynthesis (in the form of substrate dichotomy between desaturation and elongation) but also species-specific bottlenecks as exemplified by the counter-productive PDAT activity observed in linseed (see Fig. 1 for details).

The collective studies of Qi et al. (2004) and Abbadi et al. (2004) represent a major step forward in the production of VLC-PUFAs in transgenic plants, and the significance of these advances has been recognized and discussed in a number of recent reviews (Domergue et al. 2005a, Green 2004, Napier et al. 2004a, b, Singh et al. 2005). However, it is clear that while these studies represent important biotechnological breakthroughs, they also provide some new insights into the biochemical pathways under manipulation and provide useful new tools for the dissection of the underlying enzymatic reactions.

The production of DHA in transgenic plants

Having demonstrated the accumulation of the C_{20} PUFAs in transgenic plants, it is obvious that the next step is to seek to reconstitute the DHA biosynthetic pathway. As described above, although mammals synthesize DHA from EPA via β-oxidation, many aquatic microorganisms use the simpler Δ4-desaturase route. The two activities required for this route (Δ5-elongase, Δ4-desaturase) have recently been cloned and functionally characterized, allowing for the evaluation of this pathway in transgenic plants (Meyer et al. 2004, Pereira et al. 2004, Qiu 2003). Several studies on the accumulation of DHA have very recently been published, affording some additional insights into our understanding of VLC-PUFA biosynthesis. For example, Robert et al. (2005) reported low but significant accumulation of DHA (0.2–0.5% of total fatty acids) in transgenic arabidopsis seeds. To achieve this, the authors adopted a two-step approach, using seed-specific expression of a bifunctional Δ6/Δ5-desaturase from zebrafish (Hastings et al. 2001) in conjunction with the Δ6-elongase PEA-1 from *Callomyia elegans* (Beaudoin et al. 2000) to generate EPA for subsequent elongation and desaturation to DHA by enzymes encoded by genes from the DHA-synthesizing alga *Pavlova salina*. Interestingly, Robert et al. (2005) reasoned that by using the zebrafish desaturase (which is predicted to be an acyl-CoA desaturase, rather than glycerolipid-dependent), they might overcome the problem of substrate dichotomy, using exclusively acyl-CoA-dependent reactions for the synthesis of EPA (Abbadi et al. 2004). Perhaps surprisingly, relatively low levels of ARA and EPA were seen to accumulate in transgenic arabidopsis expressing the zebrafish desaturase and *C. elegans* elongase (<5% of total).

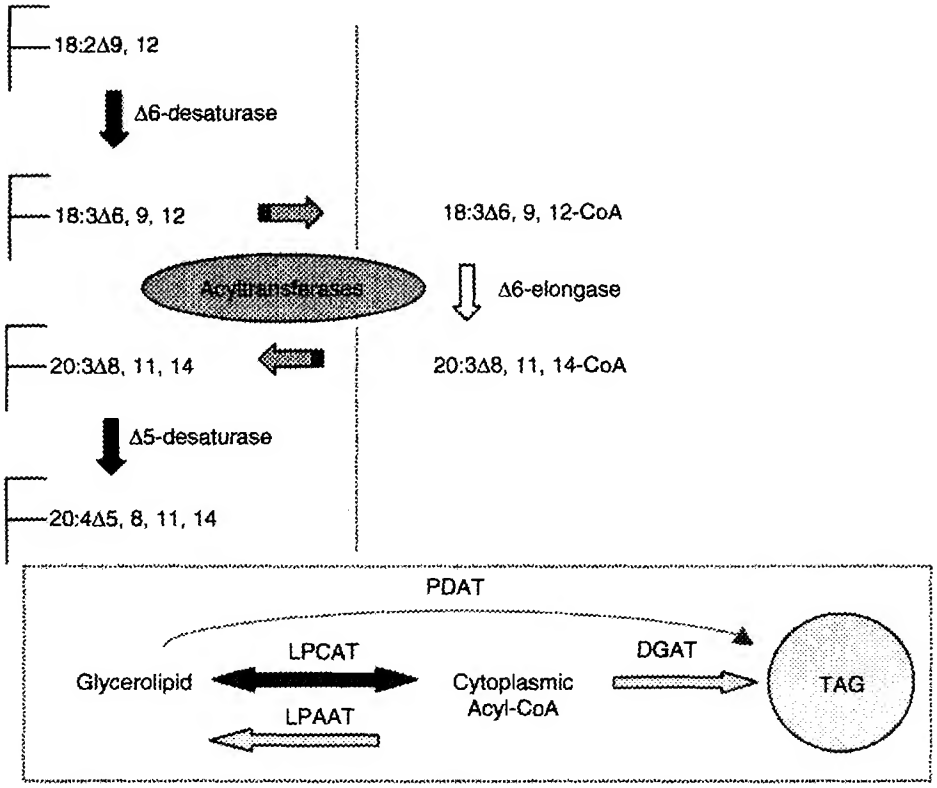

Fig. 1. The role of acyl-exchange in the biosynthesis of VLC-PUFAs. The substrate dichotomy between glycerolipid desaturation and acyl-CoA elongation is represented, exemplified by the synthesis of ARA from LA by the conventional Δ6-desaturase/elongase pathway. Glycerolipid desaturation reactions are represented by a solid black arrow, acyl-CoA-dependent elongation with an open arrow. The pathway requires acyl-exchange between the glycerolipids and the acyl-CoA pool, mediated by acyltransferases. Some activities (such as lyso-phosphatidyl acyltransferases, LPAAT; lyso-phosphatidycholine acyltransferases; LPCAT) may facilitate acyl-exchange in favour of VLC-PUFA biosynthesis, whereas others (such as phospholipid diacylglycerol acyltransferases, PDAT; diacylglycerol acyltransferase; DGAT) hinder the biosynthetic pathway by acylating potential substrates directly into triacylglycerol (TAG).

This may indicate low endogenous levels of substrate LA and ALA CoAs in arabidopsis seeds or may also imply that these animal enzymes worked poorly in transgenic plants. However, we have observed the efficient elongation of exogenously supplied GLA (in the form of a sodium soap) to transgenic arabidopsis expressing the *C. elegans* PEA-1 activity (F. Beaudoin and J. Napier, unpublished), which may indicate (residual) problems with acyl channelling between the desaturase and the elongase. Other possible considerations are that while the zebrafish desaturase is bifunctional, it is predominantly a Δ6-desaturase with some additional Δ5-desaturase activity (Hastings et al. 2001); moreover, while it is assumed that this desaturase utilizes acyl-CoA substrates, this has not been biochemically proved. Finally, the transgenic activity of the PEA1 Δ6-elongase was unexpected in that it was capable of elongating EPA to DPA; such an activity was not previously observed in our characterization of this activity in yeast (Beaudoin et al. 2000) and may represent some perturbation to optimal elongase functionality.

While the levels of DHA in transgenic arabidopsis reported by Robert et al. (2005) represented a significant proof-of-concept, two other recent studies provide more definitive data as to the feasibility of producing fish oils in transgenic plants. The first of these reports predates the study of Robert et al. (2005), in the form a patent application by Kinney et al. (2004). These researchers used a similar approach to that of Robert et al. (2005), expressing genes encoding components of the conventional Δ6-desaturase/elongase pathway in transgenic soybean seeds and somatic embryos. However, to maximize the accumulation of *n-3* VLC-PUFAs such as EPA and DHA, an ω-3 microsomal desaturase (isolated from the fungus *Saprolegnia diclina*) was also coexpressed, with the aim of converting any *n-6* VLC-PUFAs to the *n-3* form. Expression of the Δ6-desaturase, Δ6-elongase, Δ5-desaturase and the ω-3 desaturase yielded almost

20% EPA in the seeds of transgenic soybeans, as well as the unexpected accumulation of DPA in a manner similar to that observed by Robert et al. (2005). Because the Δ6-elongase utilized by Kinney et al. (2004) was from a quite distinct organism (*Mortierella alpina*) to the *C. elegans* PEA1 elongase, this apparent promiscuity is worthy of further investigation. Moreover, because neither *C. elegans* nor *M. alpina* synthesize DPA or DHA, it seems unlikely that these transgenic activities represent previously unreported specificities of the heterologous elongating activities.

Building on the successful accumulation of EPA, Kinney et al. (2004) added the two additional genes for the Δ4-pathway and observed relatively low but significant accumulation of DHA (3.1% of total fatty acids) in somatic embryos. In that respect, it is tempting to speculate that these reduced levels (compared with the levels achieved for C_{20} VLC-PUFAs) represent continued problems with substrate dichotomy between the Δ5-elongase and the Δ4-desaturase, although why similar problems are not encountered in the synthesis of EPA is not yet clear. It will be of considerable interest to see the levels of DHA achieved in stable transgenic soybean seeds, as well as the distribution of VLC-PUFAs in the acyl-CoA pool.

A second major study which demonstrates the highly efficient accumulation of C_{20} VLC-PUFAs in oilseeds has also been recently published (Wu et al. 2005), where transgenic *Brassica juncea* was shown to accumulate up to 25% ARA or 15% EPA. In addition, expression of activities for the Δ4-pathway for DHA synthesis resulted in low but significant amounts of DHA (0.2–1.5% of total). The strategy used by Wu et al. (2005) was similar to that used by Kinney et al. (2004) but involved more transgenes. In addition to the primary enzymes of VLC-PUFA biosynthesis, an ω-3 desaturase from *Phytophtora infestans* was introduced to convert *n-6* substrates to *n-3*, and a Δ12-desaturase (isolated from *Calendula officinalis*) also coexpressed to increase the flux through the entire transgenic pathway. In an attempt to overcome the problem of substrate dichotomy, an acyltransferase activity from *Thraustochytrium* was also coexpressed, but it is not clear if this delivered any enhancements to the biosynthesis of DHA. Interestingly, the C_{20} VLC-PUFAs ARA and EPA were found to be distributed evenly amongst all three positions in TAG, implying that these fatty acids are efficient substrates for endogenous enzymes involved in storage lipid synthesis. Collectively, the data of Wu et al. (2005) indicate that *B. juncea* is a highly efficient host for the synthesis of ARA and EPA to high levels, but as observed in the other studies, capable only of low level synthesis of DHA.

The studies of Kinney et al. (2004), Robert et al. (2005) and Wu et al. (2005) provide new insights into the potential of producing VLC-PUFAs in transgenic plants, but also raise a number of intriguing questions. For example, it is not precisely clear why *B. juncea* and soy are so much more efficient at the synthesis of C_{20} VLC-PUFAs such as ARA and EPA, when compared with linseed or tobacco (Abbadi et al. 2004). In that respect, more detailed analysis of these efficient accumulators is required to determine whether this is due to the transgenic activities or simply related to the differing contributions of endogenous acyl-channelling activities in different plant species (Fig. 1). It is clear that the differences observed in efficiency of reconstituting the same pathway into different plant species indicate that a detailed characterization of any potential host would be advisable prior to embarking on any transgenic interventions.

Perhaps, the most striking observation of the all the above studies is the relative inefficiency of DHA synthesis, independent of host species used (soy, arabidopsis, *B. juncea*); this may indicate a generic problem in the heterologous reconstitution of DHA synthesis. Even in species that accumulate EPA at high levels, elongation to DPA and subsequent desaturation to DHA are very low, implying that the endogenous activities that facilitate bypassing the C_{18-20} substrate dichotomy are unable to do the same for C_{20-22} substrates. For example, *B. juncea* may be capable of efficient EPA synthesis, because endogenous acyl-exchange enzymes such as acyl-CoA lyso-phosphatidylcholine acyltransferase (LPCAT) can recognize C_{18} Δ6-desaturated fatty acids and exchange them between the PC and the acyl-CoA pool, facilitating the transgene-mediated elongation (Fig. 1). In the case of EPA, LPCAT may simply fail to recognize this non-native fatty acid as a substrate, excluding it from the acyl-CoA pool. Given what is now known about the requirements of the synthesis of C_{20} VLC-PUFAs from C_{18} substrates, it seems likely that similar constraints arising from substrate dichotomy act as a major bottleneck in the synthesis of DHA from EPA. However, further detailed analysis of the transgenic plants described in Kinney et al. (2004) and Wu et al. (2005) is required to confirm this.

One final consideration raised by Wu et al. (2005) is regarding the efficiency of reconstitution of the heterologous VLC-PUFA elongase. It is now well documented that the microsomal elongase is a multienzyme complex, which progresses the sequential reactions required for acyl chain elongation (namely condensation, ketoreduction, dehydration and enoyl-reduction). Because the demonstration of Beaudoin et al. (2000) and Parker-Barnes et al. (2000) that heterologous expression

of presumptive ELO-like condensing enzymes could redirect the substrate-selectivity of endogenous elongases towards PUFA substrates (e.g. GLA), it has been assumed that the contribution to VLC-PUFA synthesis of the other elongase components is neutral. However, it seems possible that this too may be subjected to species-to-species variation and may need further investigation. Moreover, Beaudoin et al. (2002) demonstrated that overexpression of the elongase ketoreductase increased the accumulation of (heterologous) PUFAs in yeast, presumably by either increasing flux through the elongase or increasing the absolute number of elongase complexes. In addition, some plant families, such as the Brassicas, synthesize VLC fatty acids (although these are saturated and monounsaturated), and there may be subtle differences in their microsomal elongases which facilitate more efficient reconstitution of VLC-PUFA biosynthesis (as distinct from the above-mentioned species-specific differences in acyl exchange). Interestingly, Wu et al. (2005) used a variety of *B. juncea* which does not accumulate VLC fatty acids, analogous to low erucic acid oilseed rape (*Brassica napus*) and the *fae1* mutant of Arabidopsis, both of which are mutated in the condensing enzyme of the microsomal elongase (Millar et al. 1998). Whether mutations/ablations in endogenous condensing enzymes facilitate the more efficient reconstitution of the heterologous PUFA elongase is unclear but worthy of closer examination. It is tempting to speculate that there may be some form of titration between the endogenous and heterologous condensing enzymes, although it is important to remember that the heterologous PUFA (ELO) activities are structurally unrelated to the endogenous (FAE1-like) enzymes.

Additional activities to enhance the accumulation of VLC-PUFAs in transgenic plants

As discussed above, transgenic plants have now been shown to accumulate the C_{20} VLC-PUFAs ARA and EPA to significant levels well within (or even above) the target range by which they could be judged as successful. However, while DHA can clearly be synthesized, this occurs at a much lower level, thus additional approaches or factors need to be sought to enhance VLC-PUFA accumulation. Of particular relevance to the topic of this review is the report by Domergue et al. (2005b) of the identification of an acyl-CoA-dependent desaturase from a lower eukaryote. This Δ6-desaturase from *Ostreococcus tauri* is the first report of such an enzyme from non-animal sources and opens the prospect of identifying similar acyl-CoA-dependent enzymes responsible for Δ5 and Δ4-desaturation. This in turn would facilitate the possibility of VLC-PUFA biosynthesis being carried out entirely in the acyl-CoA pool and avoiding the requirement for exchange with PC (as proposed by Abbadi et al. 2004 and Robert et al. 2005).

A second activity that may be of use in the synthesis of VLC-PUFAs in transgenic plants is a recently characterized acyl-CoA synthetase from the DHA-accumulating microalgae *Thalassiosira pseudonana* (Tonon et al. 2005a). *T. pseudonana* synthesizes EPA and DHA by the conventional Δ6-desaturase/elongase pathway, and recently, all the desaturases and elongases required for their synthesis have been functionally characterized by heterologous expression in yeast (Meyer et al. 2004, Tonon et al. 2005b). Tonon et al. (2005a) characterized in yeast the *T. pseudonana* acyl-CoA synthetase TpLacA and found it is involved in the activation of VLC-PUFAs, specifically DHA. The authors speculated that this enzyme may play a crucial role in the incorporation of DHA into TAG, although this remains to be demonstrated in Planta.

Finally, the LPCAT enzyme whose (reverse) activity is postulated to be pivotal in mediating acyl exchange between the PC and the acyl-CoA pool (Abbadi et al. 2004, Beaudoin and Napier 2004) has recently been cloned and functionally characterized (Domergue et al. 2005a, detailed in Renz et al. 2004). The LPCAT enzyme was identified by a 'gain-of-function' assay in yeast, in which candidate acyltransferases from VLC-PUFA accumulating organisms were tested for any ability to enhance the elongation of glycerolipid-linked GLA. Thus, candidate LPCAT activities would be predicted to increase elongation, via an exchange of GLA into the acyl-CoA pool, providing a substrate for elongation (Fig. 1). Renz et al. 2004) identified an acyltransferase from *C. elegans* that satisfied these criteria and is therefore likely to represent LPCAT. It will be very interesting to assess the impact of this activity on the accumulation of VLC-PUFAs in transgenic plants, as well to further functionally characterize the biochemical properties of the LPCAT enzyme. In particular, the contribution of the (predominant) forward reaction of LPCAT (the synthesis of PC from acyl-CoA and *lyso*-PC) needs to be assessed both in yeast and transgenic plants. However, the elegant 'gain-of-function' screen used to identify the putative LPCAT activity provides a very effective approach to identifying further enhancing activities for VLC-PUFA synthesis.

Conclusions

As detailed above, several recent studies have proved beyond doubt the feasibility of using transgenic plants to

synthesize VLC-PUFAs such as ARA, EPA and DHA. This not only represents a considerable vindication of the concept of 'green factories' for the transgenic synthesis of novel fatty acids but also provides a sustainable source of these important components of human nutrition. To that end, the unquestionable benefits of VLC-PUFAs to human health mean that it is also possible that transgenic plants with this output trait may help assure the sceptics of the value of genetic modification. Finally, the advances summarized in this review represent the culmination of efforts from several research groups and demonstrate the wider utility of using transgenic plants to synthesize non-native products.

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Stepwise engineering to produce high yields of very long-chain polyunsaturated fatty acids in plants

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Very long chain polyunsaturated fatty acids (VLCPUFAs) such as arachidonic acid (AA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are valuable commodities that provide important human health benefits[1–5]. We report the transgenic production of significant amounts of AA and EPA in *Brassica juncea* seeds via a stepwise metabolic engineering strategy. Using a series of transformations with increasing numbers of transgenes, we demonstrate the incremental production of VLCPUFAs, achieving AA levels of up to 25% and EPA levels of up to 15% of total seed fatty acids. Both fatty acids were almost exclusively found in triacylglycerols, with AA located preferentially at *sn-2* and *sn-3* positions and EPA distributed almost equally at all three positions. Moreover, we reconstituted the DHA biosynthetic pathway in plant seeds, demonstrating the practical feasibility of large-scale production of this important ω-3 fatty acid in oilseed crops.

Plants have the capacity to serve as green factories for the production of novel industrial materials, nutritionally enhanced foods or pharmaceuticals, via metabolic engineering[6–8]. One goal of plant metabolic engineering is the production of high levels of VLCPUFAs in oilseed plants[9,10], which would provide a novel and cost-effective source of these fatty acids. Several pathways for the biosynthesis of VLCPUFAs exist in nature[11]. To produce VLCPUFAs in seeds, we followed the alternating desaturation/elongation pathways of n-6 and n-3 fatty acids. The two routes commence with linoleic acid (18:2n-6, LA) and α-linolenic acid (18:3n-3, ALA), respectively, followed by sequential Δ6 desaturation, Δ6 elongation and Δ5 desaturation, leading to the synthesis of arachidonic acid (20:4n-6, AA) in the n-6 and eicosapentaenoic (20:5n-3, EPA) in the n-3 pathway. The two pathways can be interconnected by a ω3 desaturase that converts AA into EPA. Further Δ5 elongation and Δ4 desaturation reactions lead to the synthesis of docosapentaenoic (22:5n-3, DPA) and finally docosahexaenoic acid (22:6n-3, DHA). The *B. juncea* breeding line 1424 was chosen as a host plant for biosynthesis of VLCPUFAs because of its high LA content and lack of erucic acid. The constructs for VLCPUFA production in seeds carried three to nine structural genes, with each gene under the control of the seed-specific napin promoter (**Fig. 1**).

The first construct (BJ3) introduced into *B. juncea* contained a Δ6 desaturase from *Pythium irregulare*[12], a Δ5 desaturase from *Thraustochytrium* sp.[13] and a Δ6 fatty acid elongase from *Physcomitrella patens*[14]. This represents the minimal set of transgenes required for the synthesis of AA and EPA from endogenous LA and ALA. RT-PCR indicated that all three genes were highly expressed in the developing seeds (data not shown). Several new fatty acids were detected in BJ3 seeds (**Fig. 2**). The most abundant was γ-linolenic acid (GLA), the Δ6 desaturation product of LA, with an average value of 27.7% of total seed fatty acids. AA, the Δ5 desaturation product of dihomo-γ-linolenic acid (20:3n-6, DGLA), ranged from 5.0% to 8.5% (average 7.3%), whereas stearidonic acid (SDA), the Δ6 desaturation product of ALA, averaged 3.1%; several other minor new fatty acids, such as 18:2n-9 (1.7%), were also present (**Table 1**). Consequently, LA content dropped dramatically from 45.2% in the untransformed control to 13.7% in transgenic seeds. Thus, the Δ6 and Δ5 desaturases functioned well, with conversion rates of 68.3% and 94.2%, respectively. The Δ6 elongase performed less efficiently, with a conversion rate of only 23.6%. The n-6 pathway appeared to be much more effective in VLCPUFA biosynthesis, perhaps not surprisingly, given that *B. juncea* oil is characterized by high LA (45.2%) and low ALA (9.7%).

To increase LA and concurrently reduce the side-product 18:2n-9, we added a Δ12 desaturase gene from *Calendula officinalis*[15] to the triple construct, producing the construct BJ4. The addition of this desaturase resulted in only a slight decrease (0.5%) of 18:2n-9. Enhanced conversion of oleic acid (OA) to LA was evident from the decrease in OA content. Although the GLA content remained similar to that in BJ3 plants, the average level of AA increased from 7.3% to 12.0%, with the highest level reaching 17.7%. EPA also increased from 0.8% to 1.3% (**Table 1**).

In view of the results from the BJ3 and BJ4 plants, where poor elongation from 18- to 20-carbon fatty acids limited the metabolic flux, we attempted to enhance elongation by adding a second Δ6 elongase from *Thraustochytrium* sp. When expressed in yeast, this elongase showed activity with both 18-carbon and 20-carbon fatty acids, but elongated GLA and SDA much more efficiently than AA and EPA (data not shown). In transgenic plants carrying this construct, named BJ5, a slight, but still significant increase in GLA elongation occurred. This in turn resulted in an increase in AA from an average of

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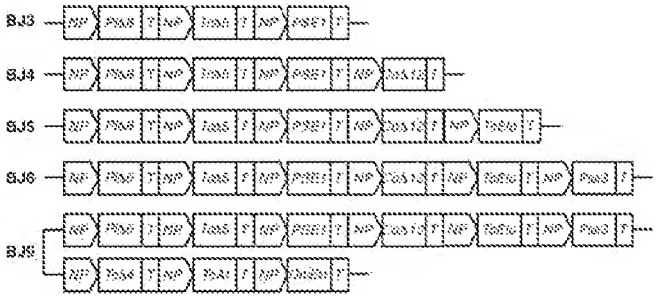

Figure 1 Simplified maps of the binary vector constructs used for plant transformation. BJ3, BJ4, BJ5, BJ6 and BJ9 represent the three-, four-, five-, six- and nine-gene constructs used for *B. juncea* transformation. *PsA6*, *TcA5*, *PSE1*, *CoA12*, *TcElo*, *Pis3*, *TcA4*, *TcAI* and *OmEl3* represent a Δ6 desaturase from *P. irregulare*, a Δ5 desaturase from *Thraustochytrium* sp. 26185, an elongase from *P. patens*, a Δ12 desaturase from *C. officinalis*, an elongase from *Thraustochytrium* sp. 26185, an ω3 desaturase from *P. irregulare*, a Δ4 desaturase from *Thraustochytrium* sp. 26185, a lysophosphatidyl acyltransferase from *Thraustochytrium* sp. 26185 and an elongase from *O. mykiss*, respectively. *NP*, napin promoter; *T*, terminator OCS.

12.0% in BJ4 to 13.7% in BJ5 seeds, with the highest value observed being 25.8% (**Table 1**). Elongation of n-3 fatty acids also increased slightly, such that the overall elongation rate of both pathways increased from 34.0% in BJ4 to 38.3% in BJ5 seeds.

The high metabolic flux via the n-6 pathway in *B. juncea* resulted in the accumulation of considerable amounts of n-6 fatty acids such as GLA and AA. To use these n-6 fatty acids for the production of n-3 fatty acids, we included a ω3 desaturase from *Phytophtora infestans* in the construct BJ6. In yeast, this desaturase introduced an n-3 double bond specifically into AA (data not shown). The ω3 desaturase also effectively converted AA into EPA in transgenic seeds. As a result, the EPA content increased significantly, from an average of 1.4% in BJ5 to 8.1% in BJ6 plants, with a concurrent decrease in AA (**Table 1**).

After achieving substantial production of AA and EPA in plant seeds, the next logical step was to produce DHA. Therefore, three more genes were added to BJ6, creating the nine-gene construct BJ9. One of these genes encodes an elongase from *Oncorhynchus mykiss* that can elongate both 18- and 20-carbon fatty acids in yeast[16], whereas the second gene encodes a Δ4 desaturase from *Thraustochytrium* sp.[15]. The third gene, also from *Thraustochytrium* sp., represents a putative lysophosphatidic acid acyltransferase. We reasoned that this enzyme from a VLCPUFA-rich organism might improve trafficking of very long chain fatty acyls among lipid pools. Transcripts from all nine genes were detected in transgenic plants (data not shown). A fatty acid with a retention time identical to that of DHA was present in BJ9 seeds (**Fig. 2**), and gas chromatography/mass spectrometry (GC/MS) analysis confirmed that this fatty acid was indeed DHA (data not shown). The average yield of DHA was 0.2% of total fatty acids, whereas the highest observed value was 1.5%. BJ9 transgenic plants also produced slightly higher levels of EPA, with the highest observed level reaching 15.0% of total fatty acids. Whether the lysophosphatidyl acyltransferase or the third elongase contributes to the improvement in EPA production remains to be determined.

Elongation of EPA appears to be a serious bottleneck in DHA synthesis. The elongation rate from EPA to DPA was only 4%; consequently, only a low level of DHA was produced in BJ9 seeds. This might be due to limitations in the host plant's ability to release EPA into the acyl-CoA pool. Alternatively, the heterologous elongase may not cooperate efficiently with the endogenous elongation complex.

It should be noted that the fatty acid profiles and the derived conversion rates reflect only the situation in the whole cell, and disregard the possible existence of unavailable fatty acid pools. For instance, in transgenic *B. juncea* GLA accumulated to a relatively stable level (27.1 to 29.4%) regardless of the construct used. These GLA molecules could be incorporated into certain lipid classes, such as triacylglycerols, where they become unavailable for further modification. Nonelongated GLA seems to remain at a constant level, with amounts exceeding this threshold apparently becoming available for elongation. This is best illustrated in BJ4 plants, where the addition of a Δ12 desaturase led to a 7.8% decrease in OA, and the consequent increase in substrate flux led to a 5.9% increase of 20-carbon VLCPUFAs, rather than in the further accumulation of 18-carbon fatty acids.

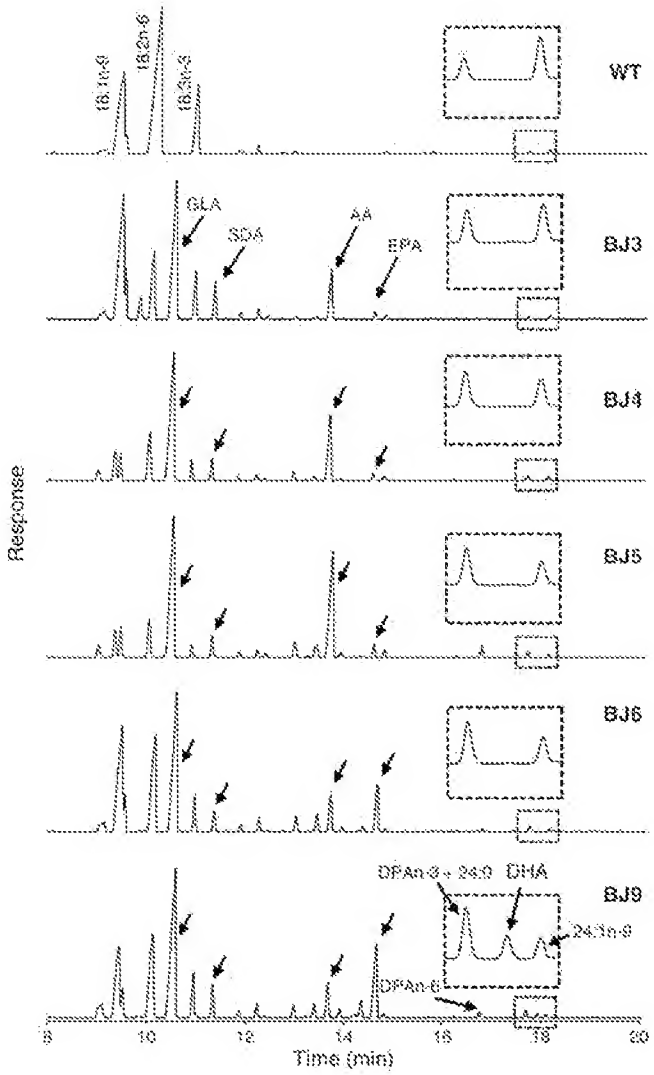

Figure 2 GC analysis of seed fatty acid methyl esters from wild-type and transgenic *B. juncea* plants. The constructs used for transformation are described in **Figure 1**. GLA, γ-linolenic acid; SDA, stearidonic acid; AA, arachidonic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; DPA, docosapentaenoic.

Table 1 Total fatty acid composition of oilseeds from the wild-type and transgenic *B. juncea* plants (wt%)

Fatty acid	Wild type ($n = 14$)	BJ3 ($N = 10$; $n = 19$)	BJ4 ($N = 7$; $n = 20$)	BJ5 ($N = 4$; $n = 28$)	BJ6 ($N = 3$; $n = 12$)	BJ9 ($N = 8$; $n = 30$)
16:0	5.6 ± 0.2	5.7 ± 0.2	5.8 ± 0.1	5.7 ± 0.1	5.6 ± 0.1	5.1 ± 0.2
18:0	1.7 ± 0.1	2.1 ± 0.1	2.2 ± 0.1	2.5 ± 0.1	2.4 ± 0.1	3.0 ± 0.2
18:1n-9 (OA)	33.2 ± 0.7	26.5 ± 0.4	18.7 ± 0.5	15.8 ± 0.9	18.4 ± 1.0	18.9 ± 1.0
18:1n-7	3.4 ± 0.1	3.5 ± 0.1	3.2 ± 0.1	3.3 ± 0.1	2.8 ± 0.10	2.4 ± 0.1
18:2n-9		1.7 ± 0.2	1.2 ± 0.2	0.5 ± 0.1	0.2 ± 0.04	0.2 ± 0.1
18:2n-6 (LA)	46.2 ± 0.5	13.7 ± 0.3	14.2 ± 0.4	15.4 ± 0.7	16.9 ± 0.5	16.0 ± 0.5
18:3n-6 (GLA)		27.7 ± 0.5	29.4 ± 0.9	28.6 ± 0.6	27.1 ± 0.8	27.3 ± 0.7
18:3n-3 (ALA)	9.7 ± 0.2	4.6 ± 0.3	4.1 ± 0.1	3.1 ± 0.2	4.2 ± 0.5	3.0 ± 0.1
18:4n-3 (SDA)		3.1 ± 0.1	2.7 ± 0.1	2.2 ± 0.1	2.4 ± 0.1	2.2 ± 0.1
20:3n-6 (DGLA)		0.5 ± 0.1	1.2 ± 0.1	2.1 ± 0.1	2.0 ± 0.1	1.9 ± 0.1
20:4n-6 (AA)		7.3 ± 0.4 (5.0–8.5)	12.0 ± 0.5 (8.2–17.7)	13.7 ± 0.7 (9.8–25.8)	5.4 ± 0.3 (4.0–7.4)	4.0 ± 0.2 (2.6–7.3)
20:4n-3 (ETA)				0.5 ± 0.1	0.6 ± 0.1	1.1 ± 0.1
20:5n-3 (EPA)		0.8 ± 0.1 (0.1–1.1)	1.3 ± 0.2 (0.9–1.7)	1.4 ± 0.1 (0.9–2.7)	8.1 ± 0.4 (6.4–11.0)	8.1 ± 0.4 (2.8–15.0)
22:5n-3 (DPA)						0.1 ± 0.02
22:6n-3 (DHA)						0.2 ± 0.03 (0–1.5)
Other	1.2 ± 0.2	2.8 ± 0.3	4.0 ± 0.2	5.2 ± 0.4	3.7 ± 0.4	6.5 ± 0.4

BJ3, BJ4, BJ5, BJ6 and BJ9 represent the transgenic plants generated from the three-, four-, five-, six- and nine-gene constructs. *N* and *n* are the number of independent transgenic plants and the total number of positive seeds analyzed, respectively. Each value represents the mean ± s.e.m. The values in brackets indicate the fatty acid ranges for AA, EPA and DHA. Other fatty acids include 14:0, 16:1n-7, 20:0, 20:1n-9, 20:2n-6, 22:0, 24:0 and others.

Transgenic BJ9 plants were phenotypically normal, as were plants carrying other constructs, and seeds containing substantial amounts of VLCPUFAs showed no obvious germination problems (data not shown). Thus, transgenic *B. juncea* seeds might be able to use the newly synthesized VLCPUFAs to support initial growth.

Although overall elongation rates in transgenic *B. juncea* appear low, they are actually much higher than those observed in flax and tobacco[10]. Accordingly, transgenic *B. juncea* accumulated higher amounts of VLCPUFAs in seeds. This could be due to the contribution of endogenous enzymes in *B. juncea* that are directly involved in the elongation process. The elongation complex includes four enzymes[17], and although the condensing enzyme (elongase) is critical in determining the substrate specificity of the elongation process, the remaining three enzymes may also play important roles in the overall elongation efficiency. Whereas erucic acid represents approximately 45% of total seed fatty acids in traditional *B. juncea* lines, the line used here contains only a trace amount of erucic acid. This might be due to a mutation in a particular condensing enzyme, as was observed in low erucic acid *Brassica napus*[18]. The remaining three enzymes in the elongation complex may thus be free to co-act with the transgenic elongase, resulting in higher elongation rates than in flax or tobacco. Alternatively, in *B. juncea* the shuffling of fatty acyls between the phospholid and acyl-CoA pools during the biosynthesis of VLCPUFAs may be more efficient. Indeed, in transgenic flax and tobacco, the low rate of elongation of Δ6-desaturated 18-carbon fatty acids to their 20-carbon counterparts was associated with a low level of Δ6-desaturated 18-carbon fatty acids in the acyl-CoA pool[10].

The VLCPUFAs produced in *B. juncea* seeds are almost exclusively present as triacylglycerols. For example in BJ4 and BJ9 seed, 93.9–98.6% of total AA and 96.0–98.1% of total EPA was found in triacylglycerols. Other lipid classes contain only very small amounts of these fatty acids. This is not unexpected, given the high levels to which AA and EPA accumulated in the seeds.

Positional analysis of BJ4 and BJ9 phospholipids (**Fig. 3a**) showed that GLA and AA were mainly located at the *sn-2* position, which appears to be the main site of Δ5 and Δ6 desaturation[19]. However, EPA, which is also a Δ5-desaturation product, was not predominantly located at the *sn-2* position. For most fatty acids, distribution at the *sn-1* and *sn-2* positions of triacylglycerol reflected the distribution pattern in phospholipids (**Fig. 3b**). The majority of AA was located at the *sn-2* and *sn-3* positions and EPA was almost equally distributed at all three positions of triacylglycerol. Preliminary analyses of developing BJ4 seeds (30 days after flowering) indicated that the fatty acid compositions of phospholipids and triacylglycerols were similar to those in mature seeds (data not shown). This suggests that the relative AA content in phospholipids is not dramatically reduced during seed desiccation, in contrast to what has been observed for medium-chain fatty acids[20,21].

A recent study reconstituted the conventional Δ6 desaturase/elongase pathway in seeds of transgenic flax and tobacco[10]. While the transgenic seeds accumulated high levels of Δ6 desaturated fatty acid, amounts of AA and EPA were only in the range of 1–2%. In work with *Arabidopsis thaliana*[9], somewhat higher levels of AA and EPA accumulated in leaves of plants carrying genes from the alternative Δ9 elongase/Δ8 desaturase pathway. This suggested that using the alternative pathway might overcome the problems associated with relatively poor elongation rates in the conventional pathway[9,22,23]. In fact, our preliminary experiments showed that the constitutive expression of the Δ6 elongase pathway in *B. juncea* resulted in even higher EPA levels in leaves than those observed in seeds (data not shown), implying that the particular host plant and targeted tissue have major effects on the efficiency of individual systems.

The synthesis of VLCPUFAs in plant seeds is an intricate biochemical process, requiring the sequential activity of multiple transgenic enzymes. The use of stepwise metabolic engineering provides an opportunity to observe the effects of individual genes in the biosynthetic pathway and offers insights into the intermediate steps of this complex process. Using this stepwise engineering, we increased the AA from 8.5% in BJ3 plants to 25.8% in BJ5 plants (maximum observed levels). The highest EPA level observed in BJ5 seeds was 1.1%, which increased to 15.0% in BJ9 plants. Since these measurements were taken from segregating populations, the highest observed value demonstrates the potential of lines to produce specific fatty acids.

The cloning of the first Δ4 desaturase from *Thraustochytrium* sp.[5] implied the existence of a simple pathway for DHA biosynthesis and suggested the possibility of producing this important fatty acid in

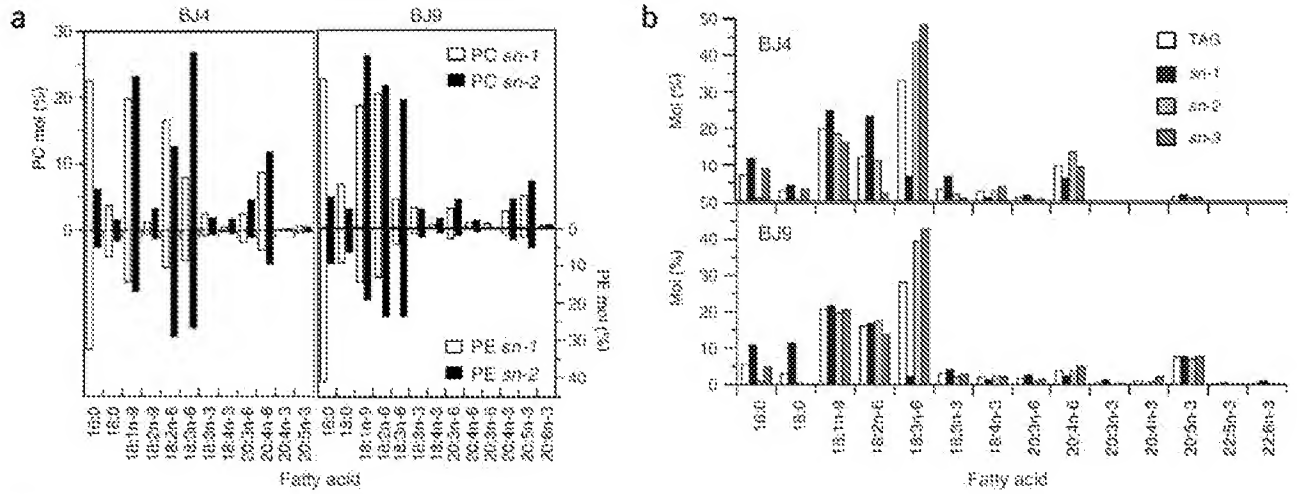

Figure 3 Stereospecific analysis of phospholipids and triacylglycerols. (**a**) The positional distribution of phosphatidylcholine (upper panels) and of phosphatidylethanolamine (lower panels). The distribution of selected fatty acids between the *sn-1* and *sn-2* positions are shown for BJ4 (left) and BJ9 (right) seeds. Fractionated phospholipids from mature seeds were digested with phospholipase A2 and the products were resolved by TLC, transmethylated and analyzed by GC. (**b**) The positional distribution of fatty acids in triacylglycerols from mature BJ4 and BJ9 seeds. Triacylglycerols were partially deacylated using ethyl magnesium bromide and the purified α,β-diacylglycerols were used in the synthesis of phosphatidylcholine. The distribution of fatty acids at the *sn-1* and *sn-2* positions was analyzed by digesting the resulting PC with phospholipase A2 and the *sn-3* position was calculated as described in the text. PC, phosphatidylcholine; PE, phosphatidylethanolamine; TAG, triacylglycerols.

plants. Here we described the reconstitution of the entire DHA biosynthetic pathway in plants. Achieving DHA synthesis in seeds, albeit at low levels, is a basis for further optimization to attain commercially viable levels, as has been demonstrated here for AA and EPA.

METHODS

Vector construction and plant transformation. A triple cassette containing three napin promoters[24], three different multiple cloning site linkers and three octopine synthase (OCS) terminators was prepared in the plasmid pUC19. A three-gene construct (BJ3) was built by inserting *PtA6*, a phospholipid-acyl Δ6 desaturase gene from *P. irregulare*[12], *TcAS*, a phospholipid-acyl Δ5 desaturase gene from *Thraustochytrium* sp.[13], and *PSE1*, an acyl CoA elongase gene from *P. patens*[14], into the multiple cloning sites (**Fig. 1**). For the four-gene construct (BJ4), an *Xba*I/*Sal*I fragment containing a phospholipid-acyl Δ12 desaturase gene from *C. officinalis* (*CoA12*)[15] linked to a napin promoter and OCS terminator was removed from a one-gene construct and subcloned into the three-gene construct. The same approach was applied to make the five- and six-gene constructs (BJ5 and BJ6) by adding an elongase gene from *Thraustochytrium* sp. (*Te3Elo*) and a phospholipid-acyl ω3 desaturase gene from *P. infestans* (*Pio3*), respectively. Finally these three-, four-, five- and six-gene constructs were removed from pUC19 by digestion with *Asc*I and cloned into the binary vectors pGPTV or pSUN2. For the nine-gene construct (BJ9), a three-gene construct containing the *TcA4* phospholipid-acyl Δ4 desaturase gene from *Thraustochytrium* sp.[13], an elongase, *OmElo*, from the fish *O. mykiss*[16] and a lysophosphatidyl acyltransferase, *TcAt*, from *Thraustochytrium* sp., was constructed and transferred into the six-gene binary vector using the Gateway system (Invitrogen). All binary vectors used the *NPTII* gene with the NOS promoter as a selection marker. Binary vectors were transferred into *Agrobacterium tumefaciens* strain GV3101 (pMP90) by electroporation.

For plant transformation, hypocotyls from 5 to 6 day old seedlings of the 0% erucic acid *B. juncea* breeding line 1424 were used as explants for inoculation with *A. tumefaciens* containing the binary constructs described above. Transformation of *B. juncea* was performed as described[25].

Fatty acid and lipid analyses. Fatty acid analyses of seeds and yeast cultures were performed by GC as described previously[17]. Individual fatty acids were identified by comparing the GC peaks with authentic fatty acid standards and/or by GC/MS.

If seeds were to be used for more detailed lipid analyses, individual seeds were first heated for 10 min at 95 °C in 1 ml of isopropanol, and after homogenization, 30-μl aliquots were removed and analyzed by GC to identify the segregating transgenic and nontransgenic seeds. The isopropanol extracts of transgenic seeds were then pooled (12 seeds per sample), centrifuged, the supernatant collected and the pellet reextracted with isopropanol/chloroform 1:1 (vol/vol). The two extracts from each sample were combined, evaporated and dissolved in chloroform. T2 seeds were processed directly without GC screening. The resulting lipid extract was prefractionated into neutral lipids, glycolipids and phospholipids on a silica PrepSep column (Fisher Scientific)[26]. These fractions were further resolved on silica G-25 thin layer chromatography (TLC) plates (Macherey-Nagel). Neutral lipids were developed with hexane/diethyl ether/acetic acid (70:30:1, vol/vol/vol), glycolipids with chloroform/methanol/ammonia (65:25:4, vol/vol/vol) and phospholipids with chloroform/methanol/ammonia/water (70:30:4:1, vol/vol/vol/vol). The individual lipid classes were identified under UV light after a primuline spray (0.05% (wt/vol) in acetone/water, 80:20, vol/vol; Sigma), removed from the plate by scraping, and used for direct transmethylation or extracted by an appropriate solvent for further analysis. The diacylglycerols were extracted and resun on a boric acid–containing silica TLC plate with chloroform/acetone (96:4, vol/vol) before GC analysis.

Positional analysis of triacylglycerols and phospholipids. Separated and extracted phospholipid classes were dissolved in 0.5 ml of borate buffer (0.5M, pH 7.5, containing 0.4 mM $CaCl_2$). After a brief sonication, 50 of phospholipase A2 from venom of *Naja mossambica* (Sigma P-7778) and 2 ml diethyl ether were added and samples were vortexed for 2 h at 22 °C. The ether phase was evaporated, the digestion was stopped with 0.3 ml 1M HCl, and the reaction mixture was extracted with chloroform/methanol (2:1, vol/vol). The digested phospholipids were separated by TLC in chloroform/methanol/ammonia/water (70:30:4:2, vol/vol/vol/vol) and spots corresponding to released free fatty acids and lysophospholipids were removed by scraping and directly transmethylated.

Fatty acid profiles of triacylglycerol stereopositions were determined by partial chemical deacylation of 20–30 mg of TLC-purified triacylglycerol, as described previously[10], with some modifications. α,β-diacylglycerol was purified by TLC on boric acid–treated silica plates (chloroform/acetone, 96:4, vol/vol), extracted and used for phosphatidylcholine synthesis[27]. The mixture of

phosphatidylcholine molecules with head groups at *sn-1* and *sn-3* positions was extracted from silica after TLC (chloroform/methanol/ammonia/water, 70:30:4:1, vol/vol/vol/vol) and digested with phospholipase A2 as described above for phospholipids. The fatty acid profile of the resulting lysophosphatidylcholine represented the triacylglycerol *sn-1* position, and the released free fatty acids, the *sn-2* position. The remaining *sn-3* position was calculated according to the formula $sn\text{-}3 = 3 \times \text{TAG} - (sn\text{-}1 + sn\text{-}2)$.

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COMPETING INTERESTS STATEMENT

The authors declare competing financial interests (see the *Nature Biotechnology* website for details).

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None